REFECTION IN THE RAT[1]

WITH AN APPENDIX ON METHODS OF PREPARING BASIC MATERIALS FOR DEFICIENT DIETS

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EIGHT FIGURES

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HISTORICAL

Refection was first reported from the Copenhagen Laboratory of Fridericia in 1926, and was observed independently by Roscoe ('27).

Refection refers to a condition in experimental animals by virtue of which they are able to grow and reproduce, even in the second generation, on diets believed to be free of the undifferentiated vitamin B (Fridericia, '26).

If one is to accept the phenomenon of refection as showing what it purports to do, it must be assumed that refected animals are capable of vitamin B synthesis. Another feature of this condition is the presence of bulky, white feces in the rat. The bulk is due to occluded air, and the whiteness is due to the presence in the feces of undigested starch grains. It has been stated that these animals do not digest raw starch in normal fashion, even though the starch grains, once they have been separated from the white feces, can then be digested under appropriate conditions. Curiously enough, the feces from refected rats contain amylase, along with undigested starch.

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The subject has been well reviewed in Brownings' excellent monograph on the vitamins ('31).

The original claim of Fridericia that there is such a condition as refection has been confirmed at the Lister Institute in London by Roscoe ('27), in Hopkins Laboratory at Cambridge by Kon and Watchorn ('27), in the veterinarian-physiological laboratory at Leipzig by Scheunert and collaborators ('29), and at the Pasteur Institute of Burmah by Taylor and Thant ('29).

Two publications on refection have appeared in the American literature. Mendel and Vickery ('29) dismissed the subject in a short negative report. From their paper one gains the impression that they felt that those who claim refection in animals may have been working with diets that were less deficient in vitamin B than supposed. After our work had been in progress for a year, a second American publication confirmed the European work. In a very short statement, Parsons, Kelley and Hussemann ('33) state that "Bulky white feces containing numerous starch grains, similar to feces reported by others as typical of refection, occurred spontaneously in individual rats on a vitamin G-low ration containing raw potato starch, etc." This is the only work from this country that we have been able to interpret as confirmation of the work of Fridericia.

The one unsuccessful attempt at obtaining refection was that of Mendel and Vickery ('29), and so far as we know these last two papers represent the only contributions to the subject of refection from the United States.

DEFINITION

Because refection has been adequately reviewed (Browning, '31) with the appearance of but few papers since Browning's monograph, we will not attempt a complete discussion of the subject. Briefly, refection in the rat carries with it the idea that vitamin B is synthesized, most probably in the lower gut. Co-existing in the feces of refected rats are considerable quantities of undigested raw starch as well as amylase which can be shown to be active on fresh raw starch. The starch

which has been separated from refected feces can be digested by starch-splitting enzymes if it is given a preliminary treatment with acid alcohol, this extraction presumably removing something which is there as a coating on the grains, and which in the rat had prevented the digestion of the starch in the first place. One rat may refect another by contagion, and, whatever the refectious agent may be, it appears to be destroyed when subjected to rigid heat sterilization. Vitamin B synthesis and sub-normal starch digestion seem to go hand-in-hand; neither one appears separately.

It is reported that refection can be obtained only with raw starch in the diet (Roscoe, '27). Cooked starch or other soluble carbohydrate will not allow of refection. The ease and certainty of obtaining refection varies with the kind of raw starch fed. Refection is more difficult with corn starch, whereas it frequently occurs with rice starch, and seems most certain to occur with potato starch.

ATTEMPTS AT CONFIRMATION

We began our experiments with the idea of confirming or disproving refection. Our first effort was to attempt to obtain refection. After we had been successful in this respect our chief aim was to find out whether or not the successful growth of rats on diets containing raw potato starch, and very low in vitamin B, could be explained on the basis of unaccounted-for vitamin-B contamination of the starch.

EXPERIMENTAL

A. An attempt to confirm the work of Fridericia on refection

Experiment 1. The diets were copied closely from those used by Fridericia in order to determine whether or not we could confirm his results.

Our control rat received the following diet:

	Per cent
Casein	20
Sucrose	52
Butter fat	15
Agar	3
Salt[1]	5
Yeast	5

[1] McCollum Davis salt mixture.

The control rat I grew normally, see figure no. 1. Rat V received this diet without the yeast supplement—and the extra 5 per cent thus available to the diet was added to the casein portion, making the percentage of casein 25 instead of 20.

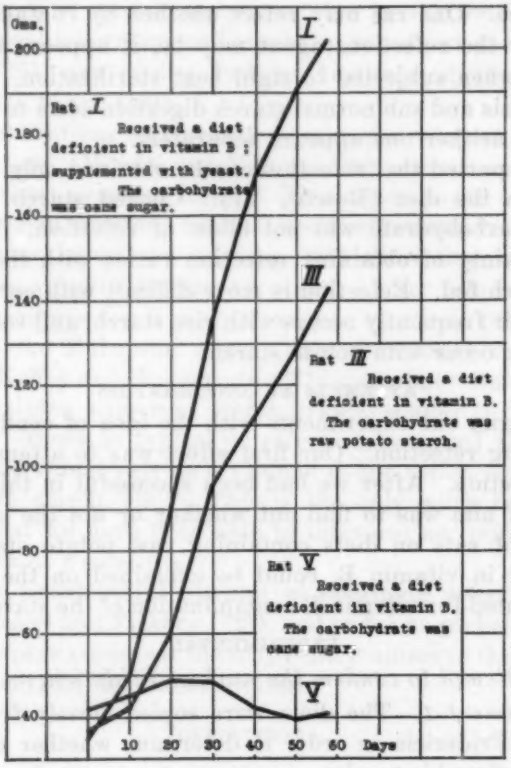

Figure 1

This rat, V, declined in weight, and died with typical symptoms of vitamin-B deficiency (fig. 1). Rat III of this series received the same diet as the other deficient rat (V) except that in the case of the carbohydrate, raw potato starch was substituted for cane sugar. The subsequent history of this

animal is essentially that of a normal animal. It grew so well and appeared in such excellent condition that it was indistinguishable from the normal control (I) receiving 0.5 gm. of dried yeast daily.[2] Examination of the growth curves leaves no doubt that, after an initial period of lag during which refection becomes established, there was normal growth in rat III in addition to general well being, even though it received a diet as devoid of vitamin B as the other rat (V) which failed to grow, with the sole exception of whatever difference, if any, there may have been between the vitamin-B content of raw potato starch and sucrose.

In addition to the favorable weight response, we also had the other criteria of refection—namely, white, bulky feces, an increase in the number of pellets and meteorism. We are convinced that we have here reproduced the phenomenon of refection.

B. Is refection due to accidental feeding of vitamin B as a contaminant of raw potato starch?

We have attempted to solve this problem in the following ways:

1. Feed one group of rats a diet containing yeast added to a vitamin-B deficient diet in which cooked starch is the carbohydrate. To another group feed an exactly similar diet with the difference that the yeast is added to the starch before cooking. If both groups show essentially the same growth, we are justified in concluding that the cooking of the starch under controlled conditions does not destroy significant amounts of vitamin B that may be present. By inference then, if added vitamin B is not destroyed in the cooking of the starch, we may assume that the cooking does not destroy the vitamin which it has been postulated may be a contaminant

[2] All rats received 2 drops of cod liver oil daily. From the data on food intake and the percentage of yeast in the diet, it is seen that this rat received what is considered an adequate amount of yeast, namely, 0.5 gm. dry yeast daily. In this experiment we thought it important to follow the details of Fridericia's original experiments as closely as possible, but in subsequent experiements we fed the supplements separate from the diet.

of raw starch. If, as indeed it turned out to be, the cooking destroys no vitamin B, the failure of rats to grow on cooked starch cannot be laid to destruction of B in the process of cooking.

We have convinced ourselves that our process of cooking starch leaves the vitamin-B content essentially unchanged (experiment 2, fig. 2).

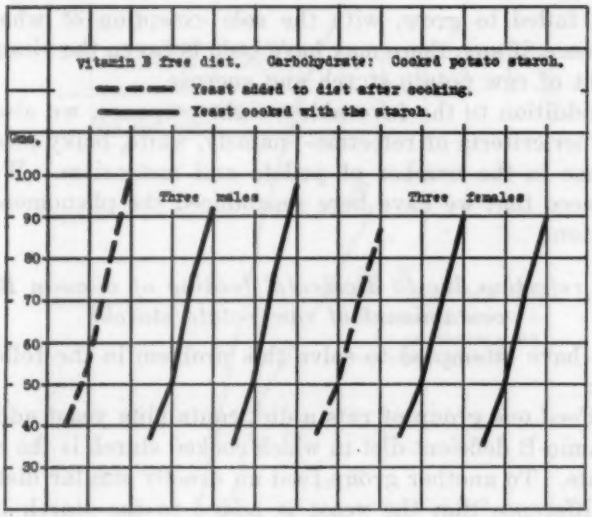

Figure 2

In section A, experiment 1, we used, as stated, a diet made up exactly as used by Fridericia. In all other experiments we made made the following changes—the yeast was added as a separate supplement to the diet—and the casein percentage was correspondingly increased from 20 to 25 per cent of the diet.

Experiment 2. The paired feeding method was used in this experiment. All six rats had identical food intakes.

Figure 2 indicates that cooking starch with yeast added before cooking gives as good a growth response as the adding

of yeast to starch after the cooking. We conclude, therefore, that growth obtained with cooked starch may be compared directly with growth with raw starch as far as the vitamin-B content of the starch is concerned.

2. Secondly, if the growth of refected rats on raw potato starch is to be attributed to vitamin-B contamination of the starch, the growth response should be uniform when no other variable factors are introduced. This is not the case. Experiment 3 (fig. 3) shows growth curves for two of our animals on a vitamin-B deficient diet, indicating the variability in the growth response when all animals are being fed from

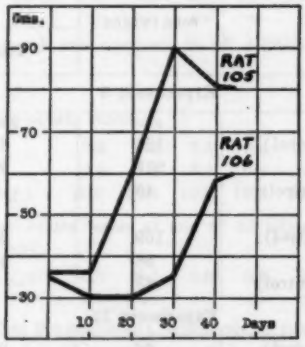

Figure 3

the same lot of diet. Obviously, if the growth obtained was due to vitamin-B contamination of the raw starch, all growth curves should be similar. Rat 105 made rapid weight gains from the tenth to the thirtieth day of the experiment, while rat 106, fed from the same lot of diet, remained stationary in weight. From the thirtieth to the fortieth days of the experiment, the positions were reversed; rat 105 declined in weight while rat 106 gained markedly. These data are representative of other similar results which are omitted for brevity. In order to check all the other constituents of the diet, aside from starch, for the lack of vitamin B we have run continuous deficient controls with cane sugar as carbohydrate

to make sure that our diets are continuously vitamin-B deficient.

In table 1 are assembled some of our data on the gains in weight achieved per gram of food eaten. In general, the positive controls (yeast added to vitamin-B deficient diets) and the refected rats (vitamin-B deficient diets with raw potato starch as the carbohydrate) gained just under 0.5 gm. in body weight per gram of food eaten. Negative controls either lost weight or made insignificant gains. The apparent variability in the

TABLE 1

	FOOD INTAKE	GAIN IN WEIGHT	
		Total	Per gram of food eaten
Experiment 5			
	gm.	*gm.*	*gm.*
Rat 36 (positive control)	128	61	0.47
Rat 37 (refected)	217	96	0.44
Rat 38 (negative control)	49	6	0.12
Rat 34 (positive control)	169	66	0.40
Rat 33 (refected)	86	48	0.55
Rat 35 (negative control)	43	— 3	— 0.07
Experiment 12			
Rat 89 (positive control)	64	26	0.40
Rat 91 (refected)	73	34	0.46
Rat 93 (negative control)	14	— 5	— 0.36

amount of food eaten and the gains made by rats under comparable regimes is explained as follows: the data have been used for just the time during which the rats were refected—and as has been shown before by others, the time of onset of refection varies.

C. Examination of the starch of refected feces

1. Separation of raw starch from feces. The dried feces were suspended in water in a cylinder. By successive decantations the starch grains were separated from other materials in the suspension, by their more rapid sedimentation.

2. Salivary digestion. Boiled starch. Using human saliva and boiled starch solutions, incubated at 40°C., we followed the disappearance of the blue color of the starch-iodine reaction as a measure of digestion. A comparison of pure potato starch (such as we used in our diets) with that recovered from refected rat feces showed a complete digestion of both

TABLE 2

Pancreatic digestion

1. Control on activity of enzyme preparation

Activity with boiled potato starch as substrate

Enzyme (cc.)	0.6	0.5	0.4	0.3	0.2	0.1	0.05
H_2O (cc.)	0.0	0.1	0.2	0.3	0.4	0.5	0.55
Starch (cc.)	0.6	0.6	0.6	0.6	0.6	0.6	0.6

Incubate at 40°C., cool in ice water at end of 30 minutes.

N/1200 iodine (cc.)	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Blue color	—	—	—	—	—	—	+

Digestion of boiled starch was complete in all dilutions from 0.6 cc. down to 0.1 cc. enzyme.

2. Activity with raw pure potato starch

Enzyme (cc.)	0.6	0.5	0.4	0.3	0.2	0.1	0.05
H_2O (cc.)	0.0	0.1	0.2	0.3	0.4	0.5	0.55
Dry starch[1] (mg.)	10.0	10.0	10.0	10.0	10.0	10.0	10.0

Incubate at 40°C., cool in ice water at end of 30 minutes, and boil to dissolve any undigested starch; cool.

N/1200 iodine (cc.)	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Blue color	—	—	—	—	±	+	+

It is seen from this that the enzymatic hydrolysis of raw potato starch is slower than with cooked starch, but digestion is certainly obtained.

3. Activity with raw starch from feces of refected rats

Enzyme (cc.)	0.6	0.5	0.4	0.3	0.2	0.1	0.05
H_2O (cc.)	0.0	0.1	0.2	0.3	0.4	0.5	0.55
Dry starch (mg.)	10.0	10.0	10.0	10.0	10.0	10.0	10.0

Incubate at 40°C., cool in ice water at end of 30 minutes, and boil to render any undigested starch detectable with iodine.

N/1200 iodine (cc.)	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Blue color	—	—	—	—	±	+	+

[1] Ten milligrams of dry starch is the amount of starch used in the previous test where 0.6 cc. of starch solution was used.

in 10 minutes. The digestion was more rapid with pure starch than with the starch from refected feces (pure starch digestion was complete in 2.5 minutes). In this connection we should state that our starch from feces was not pure white in color, but we considered it essential that we do nothing more drastic in separating the starch from the feces than suspension in water, to rule out the possibility of our removing from the grains the supposed 70 per cent alcohol-soluble coating described by Fridericia.

Raw starch. Salivary digestion was negative with both raw starches as long as it was followed (60 minutes).

3. Pancreatic digestion. The entire pancreas, and a small section of duodenum, from a freshly killed adult normal white rat was ground up with distilled water, filtered through cloth, and used in 1 per cent suspension. Such preparations are very active.

Using the Whohlgemuth method as a guide, the starch-splitting effectiveness of the preparation was tested in various concentrations of enzyme at 40°C. for 30 minutes (table 2).

It is seen that the raw potato starch separated from refected feces is digested by pancreatic enzyme preparations in a manner exactly similar to the digestion of pure raw potato starch. These experiments were repeated, introducing the single variation of doubling the amount of starch used as substrate, and the digestion of 20 mg. of raw starch from the feces of refected rats was identical with that for 10 mg. There is no escape from the conclusion that in these experiments raw potato starch from refected feces was just as readily digested as pure starch.

DISCUSSION

There is no question but that there is such a thing as refection in rats—and it must be carefully controlled in any biological assays of vitamin B. We have found in the literature what appears to be a case of refection in humans. Langworthy and Deuel ('20) in studying the digestibility of raw potato starch state that, "During this diet practically all the

subjects noted a very excessive formation of gas and frequent intestinal cramps. The quantity of feces voided was very large; a large amount of undigested starch was visible and a strong positive reaction was given with iodine." This is certainly a picture resembling the white, bulky, gas-occluded feces in the refected rat. These authors also state that, "The ingestion of the potato starch caused disagreeable physiological disturbances not noted in the other experiments with raw corn and wheat starches."

We feel it imperative that the accidental feeding of vitamin B as a contaminant of any food constituent must be ruled out. Our continuous and numerous negative controls throughout the whole time of experimentation in which vitamin-B deficiency was always obtained when cane sugar and corn starch were the carbohydrate components of the diet, justify the view that our diets are really deficient in vitamin B, and when other carbohydrates are substituted for these, there is the necessity of checking the vitamin-B factor of that component only.

The influence of coprophagy on the results has been considered (Page, '32; Guerrant and Dutcher, '32). We are aware of devices intended to prevent coprophagy. We believe that there is no device which can certainly prevent it. If a coprophagy harness is used to prevent animals from ingesting their own feces just as they emerge, they are still able to eat the feces which stick to even very large-mesh flooring of cages, because refected feces are bulky, moist, and stick well to all cage surfaces.

We have run numerous series of rats on varying yeast supplements, and find that growth curves are noticeably lowered when the 0.5 gm. daily supplement is reduced to 0.4 gm. or less. Inasmuch as we do remove a certain quantity of feces from each rat each day, it cannot have re-ingested all the vitamin B excreted.

Guerrant and Dutcher ('32) supplemented their basal vitamin-B deficient diets with feces from vitamin-G deficient rats and obtained gains in weight of only 5 to 7 gm. per week.

Our refected rats gained weight as rapidly as 3 gm. a day. The conclusion from this is that whether coprophagy is practiced or successfully prevented, the refected rat is obtaining a supply of vitamin B that is unobtainable from the feces of the usual vitamin-B deficient rat, and the conclusion is inescapable that the vitamin B is synthesized by the rat under the conditions outlined. Whether the vitamin B is absorbed from the gut directly after synthesis and before the feces are

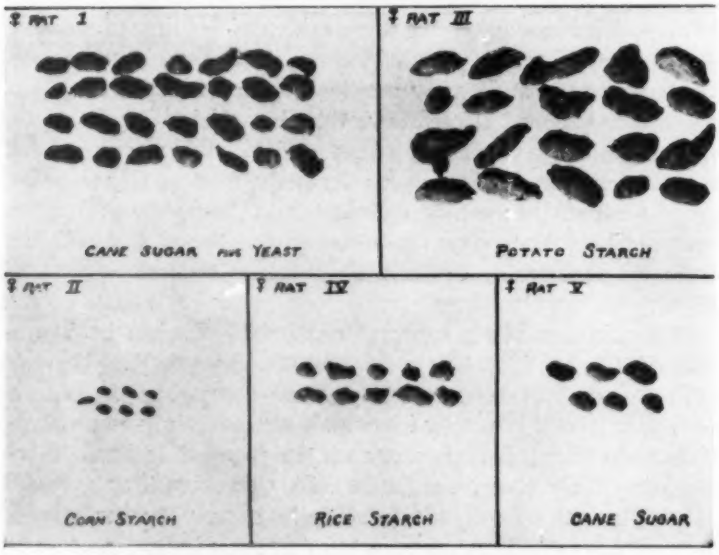

Fig. A Showing the type of feces obtained for 1 day from rat on the respective diets of raw starch (potato, corn, rice) compared with diets where cane sugar served as source of carbohydrate.

voided—or whether the vitamin is obtained, at second hand, so to speak, by re-ingestion of the feces, does not become an issue in deciding whether or not there is such a phenomenon as refection.

Our experiments on the digestibility of the raw potato starch obtained from refected feces indicated no difference in digestibility in comparison with the pure raw potato starch used in the diet.

We have not discussed the recent paper of Guerrant, Dutcher and Tomey ('35) because while it is a further contribution to the subject, it is outside the scope of the present paper.

Fig. B Rat 112, 44 days old. Permanently deficient on cooked potato starch, absence of white bulky feces.

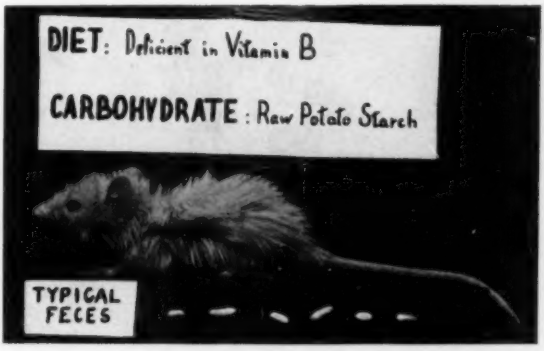

Fig. C Rat 106, 25 days old. Showing the first onset of refection, animal deficient with the first appearance of white, bulky feces which will result in the appearance of refection.

CONCLUSIONS

1. Refection has been confirmed. It can be obtained readily with diets having raw potato starch as the carbohydrate.

2. Growth of refected rats is not to be attributed to erroneously prepared diets containing unsuspected vitamin-B contaminations.

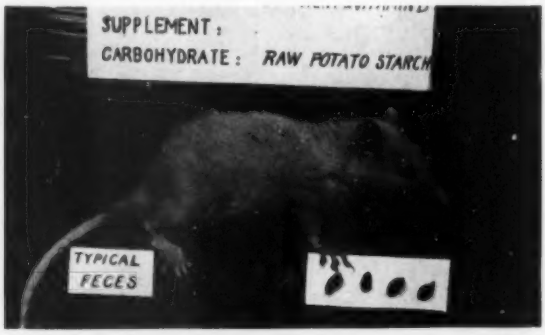

Fig. D Rat 105, 44 days old. Refected rat on diet deficient in vitamin B, showing meteorism, and both bulky white and some normal appearing feces (transition stage from deficient to refected condition). Rat normal in appearance, smaller than rat 104 because it takes a week or so for refection to become established.

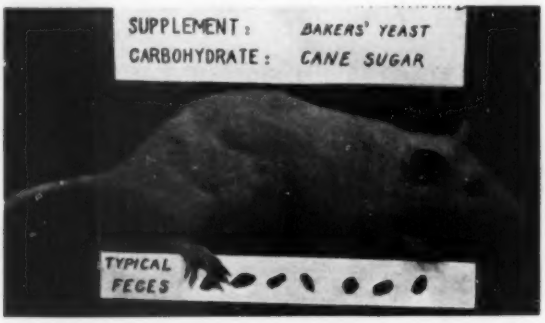

Fig. E Rat 104, 44 days old. Showing normal development on an adequate synthetic diet.

3. Raw potato starch from refected feces, and pure raw potato starch behave similarly in 'in vitro' digestion experiments with pancreatic digestion mixtures.

APPENDIX

Preparation of basic materials for diets deficient in the vitamin-B complex

Purification of commercial casein. Transfer 500 gm. casein to a 6-liter Florence flask; add 0.1 per cent acetic acid (dilute 10 cc. of '99 per cent to glacial' acid to 10 liters of distilled water) until the volume of supernatant acid about equals the volume of moist casein, allowing time for swelling of the casein. There will be enough room in the flask for adequate mixing. Stopper the flask and shake vigorously and frequently for about 4 hours. Allow casein to settle rather firmly and remove the acid wash fluid by decantation. The casein is washed in this manner twice daily for 7 days; each alternate wash stands overnight, and is given a mixing on the following morning before removing the fluid by decantation. When it is inconvenient to change the wash fluid twice in the same day, fourteen separate washings are considered equivalent, the periods to be not less than as given. Filter off the last acetic acid washing.

Return acid-extracted casein to the same flask and add 60 per cent ethyl alcohol (dilute 95 per cent alcohol with water in the ratio of 100 cc. of alcohol to 58 cc. water) bringing the mixture of casein and alcohol up to the same total volume used in the acid extraction. This alcohol extraction is continued for a full 24-hour period—with frequent shaking during the day. The casein receives three such extractions, using filtration instead of decantation after each extraction. One additional and similar extraction is made with 95 per cent alcohol. Filter to near dryness and extract for one similar period with ether.

After filtration from ether, spread out on glazed paper to air dry. When dry, only that part of the casein is used which easily passes a 40-mesh sieve, because we feel that lumpy material may have escaped adequate and uniform treatment. The final product is a finely granular and completely white powder.

Preparation of raw potato starch from potatoes. Starting with 5 pounds of potatoes, peel and remove the eyes completely. After washing, grind very finely in a food grinder. Place mash in a closely woven cotton sack and tie the sack so as to leave sufficient space for subsequent kneading.

Immerse in a pail (enameled) of clear tap water and knead vigorously with both hands until the extraction seems about complete. Kneading under the surface of the water insures appreciably higher yields. Transfer to a glass precipitating jar and pour off the liquid with all suspended material after the starch has stratified on the bottom (about 2 hours).

The moist starch is then suspended in tap water and mixed well. After settling of the starch, the liquid (containing suspended impurities) is decanted. This is continued until the washings are entirely clear. (If the starch is left wet for any extended period, a little chloroform is added.)

The starch is now suspended in water, and the suspension is poured through a 40-mesh, then through a 60-mesh, and finally through an 80-mesh sieve.

After decanting this last wash, which should be clear, spread out the starch on glazed paper to dry. To avoid damage to individual starch grains, the dried product is not screened, but left in a lumpy condition.

Separation of butter fat from butter. Place 2 pounds of butter in a beaker and heat to approximately 50°C. in a water bath until melted.

Transfer to a 2-liter separatory funnel (placed in a ring stand over a source of heat such as will maintain a temperature of about 50°C. in the funnel) and allow to remain until the butter fat separates out (about 1 hour).

Draw off and discard the emulsion in the water layer. Add distilled water (at 50°C.) to the butter fat in the funnel in such amount as will permit of gentle rotary mixing. Washing of the butter fat is accomplished by such frequent additions of water until, after mixing with the butter fat, the water phase is free of turbidity (about 5 to 6 liters of water are usually required). The butter fat receives no further

treatment, and is placed in a glass vessel in the refrigerator. (Our refrigerator maintains a temperature of a few degrees above freezing.) Butter fat, prepared in this way, will keep for months without noticeable deterioration. We have found that it is inadvisable to start with pasteurized butter, due to the difficulty of breaking the emulsions of butter fat.

Preparation of agar. Plain, granular agar is refluxed at boiling temperature with 95 per cent ethyl alcohol. Our impression is that the relative amounts of agar and alcohol may be varied with impunity.

Preparation of dried yeast. Fleishman's bakers' yeast is obtained in pound bricks. Crumble yeast with the hands, spread on glazed paper, and dry at room temperature. Complete the drying on paper-lined trays in an oven at 40°C. Material which does not pass a 20-mesh sieve is discarded.

Preparation of dry diets. Rats will sort out particles of agar from a dry diet and leave this component uneaten. It has been our experience that this can be obviated largely by impregnating the agar with butter fat. Dry casein, carbohydrate and salt mixture are thoroughly mixed. Heat the butter fat and agar together (at 50°C.) and add to the mixture of other dry components. Stirring is carried to the point of obtaining what is apparently a homogenous mixture.

Preparation of diet containing cooked potato starch (78° to 84°C. for 10 minutes). Add 50 cc. of distilled water to 3 gm. of agar in a 100 cc. beaker. Place the agar mixture (beaker covered with watch glass) in a boiling water bath.

Simultaneously, immerse an 800 cc. beaker, containing 52 gm. starch and 470 cc. distilled water, in a water bath kept at a temperature just below boiling. While it is only necessary to stir the agar occasionally, the starch must be kept in constant motion until it gelatinizes at about 65°C. (This is the figure for our own potato starch; the temperature at which starch gelatinizes varies markedly with different starches.) Unless this stage of the preparation is carried out with the utmost care, lumps will form, and the cooking of the starch will not be uniform. Until the temperature of the suspension

reaches 78°C. it is advisable to stir continuously. For the stirring we hold together a thermometer and a heavy glass stirring rod flattened into a paddle at the lower end. Count time from the attainment of a temperature of 78°C. and continue active stirring for 10 minutes. During this time the starch will remain between 78° to 84°C. with the bath temperature maintained at about 98°C.

Remove both the agar and the starch from the water baths. When the agar has cooled to the temperature of the starch, mix them well by pouring the agar into the starch with stirring. When homogenous, add the butter fat and the previously mixed casein and salt mixture and stir until the mixing of the constituents is uniform. Cover with a watch glass and place in refrigerator.

If this procedure is followed closely, the batch will weigh close to 580 gm., and the dry food factor calculated as follows: since the 580 gm. of food so prepared contains 100 gm. of dry material, the amount of food consumed by a rat is calculated as the number of grams of prepared food divided by 5.8, and is recorded thus as grams of dry weight of food. Cooked starch diets are used only up to and including the fifth day after preparation.

Supplements. All rats receive 2 drops of cod liver oil daily by mouth, using a medicine dropper. Where indicated in the data, yeast is placed in a separate dish, and moistened to prevent loss by scattering. Food is given fresh daily, and the intake weighed daily.

General notes. All rats are kept in individual cages; fresh water is supplied daily, and food and water dishes are of glazed porcelain. With cooked diets, the food dishes are washed daily.

Unless there is an occasion for doing it oftener, feces are collected and the cage cleaned twice in a 5-day period. When a cage is vacated, cleaning is followed by washing with a lysol solution.

Standard cages are used with the exception of special preparation of the bottoms. The bottom pan is covered with an absorbent paper towel. Two $\frac{3}{8}$-inch mesh galvanized iron screens are cut to fit the bottom of the pan. These two are separated from each other by a distance of 5 mm., accomplished by three glass rod supports. This arrangement insures that the rat will be dry without allowing the rat to eat any paper. We make no attempt to prevent coprophagy.

Food losses through scattering are minimized by placing a glass Petri dish under the porcelain food cups. This Petri dish is held in place by two slots cut in the standard food cup holder.

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THE COMPARATIVE ANTIRACHITIC EFFICIENCY OF VITAMIN D IN IRRADIATED MILK, METABOLIZED (YEAST) MILK, AND COD LIVER OIL [1,2]

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Several investigators (Mussehl and Ackerson, '30; Massengale and Nussmeier, '30; Russell and Klein, '31; Steenbock, Kletzien and Halpin, '32; Russell, Taylor, and Wilcox, '32, '33; Bethke, Record, and Kennard, '33) have shown that the vitamin D of cod liver oil and that of irradiated ergosterol or irradiated yeast are not identical. This fact came to be established primarily through experiments which demostrated that the chicken responds less effectively to the vitamin D in irradiated ergosterol and irradiated yeast than to the rat equivalent amount of vitamin D in cod liver oil. The report of Waddell ('34) that the provitamin constituent of cholesterol was a substance different from that of ergosterol and that irradiated cholesterol was as efficient for chicks, rat unit for rat unit, as the vitamin D of cod liver oil, gave a possible new interpretation to the vitamin D problem.

The early clinical results (Hess, Lewis, and Rivkin, '30; Barnes, Brady, and James, '30; DeSanctis and Craig, '30) on infants receiving irradiated ergosterol also brought out quan-

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[2] Presented in part before the American Institute of Nutrition, April 10, 1935. Similar data were presented by Haman and Steenbock, Proc. Am. Inst. Nutrition, J. Nutrition, vol. 9, (June) 1935.

titative differences between the vitamin D of irradiated ergosterol and cod liver oil, although to a lesser degree than in case of the chicken. More recent clinical experiments dealing with the ability of vitamin D-rich milks to cure or prevent infantile rickets have yielded results which further complicate the problem. These milks have not only been reported by Hess and associates ('31) as being more efficient than other vitamin D supplements but that they also differ among themselves in efficacy, depending upon the method by which they are enriched with vitamin D. Hess and Lewis ('32, '33) reported that the vitamin D in irradiated milk was more efficacious in the cure of infantile rickets than milk enriched in vitamin D by feeding the cows irradiated yeast. On the contrary, Kramer and Gittleman ('33) and recently Wyman, Eley, Bunker, and Harris ('35), and Gerstenberger, et al. ('35) reported that the two milks were equally efficient clinically when compared on a rat unit basis.

Experimental evidence has been presented which shows that the additional vitamin D in milk or eggs resulting from the feeding of irradiated ergosterol to the cow or chicken is in the same biological form as in the supplement fed. Krauss, Bethke, and Monroe ('32) reported that the vitamin D in butterfat from cows fed irradiated ergosterol was not so efficient for calcification in chicks as an equivalent rat unit of vitamin D from cod liver oil. Likewise, McDonald and Massengale ('32) found that the vitamin D in eggs resulting from feeding large doses of irradiated ergosterol to the laying bird was not so efficient antirachitically for chicks as equivalent rat units of vitamin D from cod liver oil. Similar (unpublished) observations have been made in our laboratory. The present study was undertaken to obtain further data on the above observations and on the comparative response of the infant and chick to the vitamin D in irradiated milk and in milk from cows fed irradiated yeast.

EXPERIMENTAL

The milks came from cows which received the same basal ration of excellent quality alfalfa hay and a grain mixture made up of ground yellow corn, ground oats, wheat bran, and linseed oil meal. In addition, one group received a definite amount of irradiated yeast mixed with the grain portion of the ration. The cows were kept under winter feeding conditions and allowed a small amount of exercise each suitable day in a vegetation-free lot. The irradiated milk was produced by running the milk from the check group of cows over a small surface cooler in a thin film and exposing it to the rays of a carbon arc lamp burning C carbons. The skim milk used in the first experiment and the whole untreated milk used in the second trial were from the check group of cows, i.e., from the same supply used for irradiation. All milks were dried before electric fans at a temperature not exceeding 50°C.

The assays on birds were carried out with single-comb White Leghorn chicks of the same parent stock. They were started on the experimental rations when 1 day old. Fifteen chicks were placed in each group. All groups were brooded indoors in pens equipped with hardware cloth floors. Ash determinations were made on the individual alcohol- and ether-extracted tibiae of twelve representative birds from each group. Calcium (Clark and Collip, '25) and inorganic phosphorus (Briggs, '23) determinations were made on the serum of the pooled blood.

The dried milks used in the first trial were from the same supply of fluid milk that Gerstenberger, et al. ('35) used in their clinical studies. They were thoroughly incorporated at a 25 per cent level in a ration of yellow corn, 42; wheat middlings, 25; commercial casein, 5; calcium carbonate, 2; and sodium chloride, 1. In order to equalize the total milk solids and fats in the rations, 19 per cent dried skim milk and 6 per cent corn oil were included in the rations of lots 1 and 4. The corn oil of the latter group (lot 4) contained cod liver oil to furnish 27 International units per 100 gm. of ration.

The results summarized in table 1 show that vitamin D in metabolized (yeast) milk was not so effective antirachitically for chicks as vitamin D in cod liver oil. Although the comparison between irradiated milk and metabolized (yeast) milk was not clear cut on account of the greater intake of vitamin D in the irradiated milk lot, they nevertheless suggest that the vitamin D in irradiated milk is probably more efficient for calcification in chicks than the factor in metabolized (yeast) milk. It was thought at the start of the experiment that the two milks were approximately equal in rat unit potency; since

TABLE 1

The antirachitic efficacy of vitamin D in metabolized (yeast) milk, irradiated milk, and cod liver oil for chicks

LOT NO.	SUPPLEMENT	VITAMIN D RAT UNITS PER 100 GM. RATION	AVERAGE WEIGHT AT 5 WEEKS	BLOOD ANALYSIS		AVERAGE ASH IN TIBIAE
				Ca. per 100 cc. serum	P. per 100 cc. serum	
			gm.	*mg.*	*mg.*	*per cent*
1	19 per cent dried skim milk	0	210.6	8.87	6.73	36.51 ± 0.48
2	25 per cent dried metabolized milk	33	240.4	9.00	4.89	37.48 ± 0.54
3	25 per cent dried irradiated milk	50	266.0	11.74	7.57	47.20 ± 0.24
4	19 per cent dried skim milk Cod liver oil	27	273.8	11.36	7.19	46.92 ± 0.15

earlier preliminary rat assays on similarly prepared fluid milks indicated equal potency. However, when we observed the apparent marked difference in antirachitic efficiency in the chick, the dried milks were assayed on rats for comparative potency. The line test method indicated that the dried metabolized (yeast) and irradiated milks contained 1.33 and 2.0 International units of vitamin D per gram, respectively.

In order further to check the comparative vitamin D potencies of the above milks, they were fed in a prophylactic trial to groups of weanling rats divided evenly as to litter and sex and maintained on the Steenbock and Black rachitic ration.

Appropriate negative and positive control groups were also included. Ash determinations were made on the individual alcohol- and ether-extracted moisture-free femurs at the end of 4 weeks. The data are presented in table 2. It is evident that the irradiated milk had a greater potency than the metabolized (yeast) milk—160 mg. of the irradiated milk daily gave the same per cent of bone ash as 240 mg. daily of the metabolized. On this basis, the comparative potencies of the two milks were on the order of 2:3, which is in agreement with the line-test results.

The second chick trial was conducted to obtain a better quantitative comparison between the two kinds of vitamin D

TABLE 2

Comparative vitamin D potencies of dried irradiated milk and dried yeast milk used in first chick experiment (prophylactic)

SUPPLEMENT FED DAILY	NUMBER OF RATS	GAIN IN WEIGHT	AVERAGE BONE ASH IN FEMURS
		gm.	*per cent*
160 mg. dried metabolized milk	8	49.3	40.68 ± 0.31
160 mg. dried irradiated milk	8	51.3	42.84 ± 0.32
240 mg. dried metabolized milk	8	52.8	42.55 ± 0.32
240 mg. dried irradiated milk	8	55.3	45.18 ± 0.24
3 drops cod liver oil	7	35.1	44.57 ± 0.24
None	8	37.3	25.41 ± 0.33

milks and cod liver oil. Since in the first chick experiment 25 per cent of dried metabolized milk did not show a positive antirachitic effect, it was necessary to increase the vitamin D content of this milk without increasing the amount of total milk solids in the ration and thus introduce other complicating factors. Accordingly, a group of cows were fed sufficient irradiated yeast to produce a highly potent milk. The irradiated milk was produced as in the first trial. Whole dried milk from the check group of cows was used in place of dried skim milk in order to have a uniform intake of total milk solids and a direct comparison between the efficacy of the vitamin D in the two kinds of milk due to individual treatment.

The dried milks were first assayed on rats by the line-test and bone-ash methods so that known amounts of vitamin D could be incorporated in the chick rations. The results of the line-test showed that the dried metabolized (yeast) milk and irradiated milk contained 4.4 and 2.2 International units, respectively, per gram. The vitamin D content of the dried check milk was so low that it was considered negligible. The results of the prophylactic assay on the milks are presented in table 3. It is apparent that the dried metabolized (yeast) milk contained approximately again as much vitamin D as

TABLE 3

Comparative vitamin D potencies of dried irradiated milk and dried metabolized (yeast) milk used in second chick experiment (prophylactic)

SUPPLEMENT FED EVERY OTHER DAY	NUMBER OF RATS	GAIN IN WEIGHT	AVERAGE BONE ASH IN FEMURS
		gm.	*per cent*
None	7	31.6	25.71 ± 0.59
165.0 mg. dried skim milk	7	40.1	25.78 ± 0.72
225.0 mg. dried whole check milk	7	38.1	26.43 ± 0.49
75.0 mg. dried metabolized milk 112.5 mg. dried skim milk	7	47.4	40.03 ± 0.74
150.0 mg. dried metabolized milk 52.5 mg. dried skim milk	7	46.6	45.26 ± 0.67
150.0 mg. dried irradiated milk 52.5 mg. dried skim milk	7	47.4	41.93 ± 0.86
225.0 mg. dried irradiated milk	7	46.3	44.06 ± 0.55
3 drops cod liver oil, daily	7	41.4	46.07 ± 0.52

the irradiated milk, since 75 mg. of the metabolized milk and 150 mg. of the irradiated milk gave about the same per cent of bone ash, thus substantiating the line-test evaluations.

In the chick experiment the dried milks were incorporated in the same basal ration used in the first trial. The total solids were kept the same in all rations by replacing an equivalent amount of dried whole check milk with the milks to be assayed. Two groups receiving in addition to the basal ration containing the dried check milk 11 and 27 International units of vitamin D from cod liver oil per 100 gm. of ration

were included for comparative purposes. The cod liver oil was diluted with corn oil so that 1 per cent supplied the desired unitage. The experiment was terminated at 43 days.

Table 4 shows conclusively that the vitamin D in metabolized (yeast) milk is decidedly less efficient antirachitically for

TABLE 4

The comparative antirachitic efficiency of vitamin D milks and cod liver oil for chicks

LOT NO.	SUPPLEMENT	VITAMIN D RAT UNITS PER 100 GM. RATION	AVERAGE WEIGHT AT 43 DAYS	BLOOD ANALYSIS		AVERAGE ASH IN TIBIAE
				Ca. per 100 cc. serum	P. per 100 cc. serum	
			gm.	*mg.*	*mg.*	*per cent*
1	25.0 per cent dried whole cheek milk	0.0	311.1	8.46	6.32	37.44 ± 0.34
2	25.0 per cent dried whole cheek milk Cod liver oil	11.0	428.1	11.28	8.15	48.13 ± 0.23
3	25.0 per cent dried whole cheek milk Cod liver oil	27.0	383.4	11.49	8.43	47.73 ± 0.24
4	12.5 per cent dried metabolized milk 12.5 per cent dried whole cheek milk	55.0	337.9	9.50	6.80	38.54 ± 0.68
5	25.0 per cent dried metabolized milk	110.0	335.4	9.82	6.64	42.77 ± 0.57
6	5.0 per cent dried irradiated milk 20.0 per cent dried whole cheek milk	11.0	387.9	11.43	8.18	48.34 ± 0.34
7	12.5 per cent dried irradiated milk 12.5 per cent dried whole cheek milk	27.5	410.5	11.07	8.18	48.35 ± 0.26

the bird than the rat equivalent amount of vitamin D from cod liver oil or irradiated milk; more than ten times the rat unitage of vitamin D in metabolized milk than in irradiated milk or cod liver oil being required to give approximately the same per cent of bone ash. It is further indicated that the vitamin D of irradiated milk and cod liver oil are equally efficient. These findings are in accord with those of Haman and

Steenbock ('35) and follow the observation of Waddell ('34) that vitamin D of irradiated cholesterol and cod liver oil are equivalent for the chick. The results obtained with the metabolized (yeast) milk also substantiate the reports of Krauss, Bethke, and Monroe ('32) and McDonald and Massengale ('32) that the animal or bird does not change one biological form of vitamin D into another.

The marked difference in the response of the chick to vitamin D from the two antirachitic milks is of especial interest, since milks from the same supply were found by Gerstenberger, et al. ('35) to possess no practical difference in antirachitic efficacy for rachitic infants when fed on an equivalent rat unit basis. It appears, therefore, that the infant and chick vary greatly in their response to equivalent rat unit intakes of vitamin D in irradiated milk and metabolized (yeast) milk.

CONCLUSIONS

It required more than ten times the rat equivalent amount of vitamin D in metabolized (yeast) milk than in irradiated milk to produce the same antirachitic effect in chicks.

Equivalent rat units of vitamin D from cod liver oil and irradiated milk were equally efficient, antirachitically, for the chicken.

The data indicate that the vitamin D in milk resulting from feeding irradiated yeast to the cow (metabolized milk) is in the same biological form as that fed to the animal.

Since rat equivalent amounts of vitamin D in metabolized (yeast) milk and irradiated milk were found equally efficacious for the rachitic infant and not for the chick, it is concluded that the infant and chick vary greatly in their response to the forms of vitamin D in these two types of milk.

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THE EFFECT OF DIGESTIBILITY UPON THE AVAILABILITY OF IRON IN WHOLE WHEAT

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ONE FIGURE

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In former papers (Rose et al., '32, '34) we have called attention to the superior results in hemoglobin regeneration which we have observed when anemic rats are fed whole wheat, as compared with results on a solution of the ash of the same amount of wheat. These findings regarding the availability of the iron in the natural food appear to be at variance with those of Elvehjem, Hart and Sherman ('33), but the latter authors have made no comparison between the effect of wheat and of the same amounts of iron and copper as in the wheat in the form of ferric chloride and copper sulphate. They fed young anemic rats 3 gm. of whole wheat yielding 0.15 mg. of iron, supplementing this with 0.05 mg. of copper; but ferric chloride was fed at levels of 0.5 and 0.3 mg. of iron and copper sulphate at a level of 0.05 mg. of copper with 0.04 mg. of manganese in addition. They say, "It is readily seen that the rate of hemoglobin formation produced by the addition of 3 gm. of wheat or oats to a milk diet supplemented with copper is much inferior to that produced by 0.5 mg. of Fe or even 0.3 mg. of Fe supplied as ferric chloride." This would naturally be expected since the wheat furnished only 30 to 50 per cent as much iron as was given in the form of ferric chloride. These findings would not seem to give any indication of the relative availability of the iron in the two forms.

It has been suggested by Elvehjem, Hart and Sherman ('34), that the differences reported with regard to the efficiency of wheat in hemoglobin regeneration may be due to the degree to which the experimental animals are depleted. We have, in the present series, subjected our animals to a more severe anemia than previously, although it has been our experience that for the testing of foods it is not desirable to go too far in this direction, since animals very severely depleted show a high mortality and give other evidences of poor physiological condition. They frequently lose appetite and fail to respond as quickly to an improved diet as animals which have a slightly higher hemoglobin level and are otherwise apparently normal. In view of these difficulties with very anemic animals, it seemed to us quite likely that the failure of very young and very anemic animals to respond to the iron in wheat might be due to difficulty in digestion of wheat. Hence in this study we have used wheat predigested with Taka diastase till the starch was completely dextrinized to furnish iron in amount equal to the raw wheat (fed in the form of finely-ground flour).

In making comparisons of this sort it is desirable to have the iron intake considerably under the optimum amount for normal regeneration. We have therefore used 3 gm. of whole wheat, yielding 0.1 mg. of iron and 0.02 mg. of copper, and have paralleled the wheat experiment with one in which the same amounts of iron and copper were fed as ferric chloride and copper sulphate respectively.

Until the young were 2 weeks old the mother rats were fed our stock ration consisting of one-third whole milk powder, two-thirds whole wheat, sodium chloride equal to 2 per cent of the wheat with a few grams of lean beef daily. The third week all received whole milk powder, but the whole wheat was given only to the mothers, who were removed from their young for several hours daily to receive the wheat. Three litters of young, thirty-one animals, were weaned at 3 weeks of age and fed exclusively on fresh whole Guernsey milk, brought in glass containers directly from the cow to the laboratory.

After 33 to 38 days on this ration, twenty-four rats had hemoglobin values between 2.2 and 3.6 gm.; and four rats, values which ranged between 4.2 and 4.5 gm. The remaining three animals, which after 44 to 46 days still had hemoglobin values of over 5 gm., were discarded. One animal died 6 days after being reduced to a hemoglobin value of 3 gm., and two others with values of 3.0 and 4.2 gm. at 57 and 53 days respectively were too weak to respond when fed the supplementary source of iron and died, one after 6, the other after 3 days of supplementary feeding.

Twenty-four animals, all with hemoglobin values between 2.2 and 3.6 gm. per 100 cc. of blood with the exception of two animals which had values of 4.4 and 4.5 gm. respectively, were divided into three lots carefully matched as to litter, age, sex, weight and hemoglobin level. These were fed fresh whole milk daily for 6 weeks, lot 1 receiving in addition 3 gm. of whole wheat finely ground; lot 2, the same amount of iron in the form of 2.7 gm. of wheat predigested till no starch could be detected with iodine; lot 3, 0.1 mg. iron as $FeCl_3$ and 0.02 mg. copper as $CuSO_4$, equivalent to the iron and copper in 3 gm. of whole wheat. At the end of 6 weeks, the total gain in hemoglobin for the undigested whole wheat averaged 7.6 gm. per 100 cc. of blood; for the predigested whole wheat, 9.6 gm.; and for the mineral supplements, 7.0 gm. The data are summarized in table 1 and in the rates of hemoglobin regeneration in figure 1.

These animals required approximately 5 weeks for depletion after weaning at age of 21 days. This is longer than the period reported by Elvehjem and his associates. The difference may be due to a higher iron content of the diet used by us for the mothers. Our animals weighed approximately twice as much at depletion, and being relatively stronger, were able to consume easily and digest well 3 gm. of whole wheat daily. Nevertheless, the improved utilization of the iron when the wheat was predigested, as indicated by a 26 per cent greater gain in hemoglobin in 6 weeks, may be taken as evidence that digestibility is a factor to be considered in the utilization of iron by young anemic rats.

TABLE 1

Age, weight and hemoglobin of anemic rats fed 3 gm. whole wheat, the same amount predigested, and iron and copper to equal that in the wheat as $FeCl_3$ and $CuSO_4$

	3 GM. WHOLE WHEAT	2.7 GM. PREDIGESTED WHOLE WHEAT	0.1 MG. Fe (AS $FeCl_3$) 0.02 MG. Cu (AS $CuSO_4$)
Average age at depletion	56 days	57 days	57 days
Average weight at weaning (21 days)	33 gm.	32 gm.	32 gm.
Average weight at depletion	80 gm.	78 gm.	79 gm.
Average weight at end	206 gm.	197 gm.	161 gm.
Average hemoglobin per 100 cc. of blood at beginning	3.3 gm.	3.1 gm.	3.3 gm.
Average hemoglobin at end of 6 weeks feeding	10.9 gm.	12.7 gm.	10.3 gm.
Average gains in hemoglobin in 6 weeks	7.6 gm.	9.6 gm.	7.0 gm.

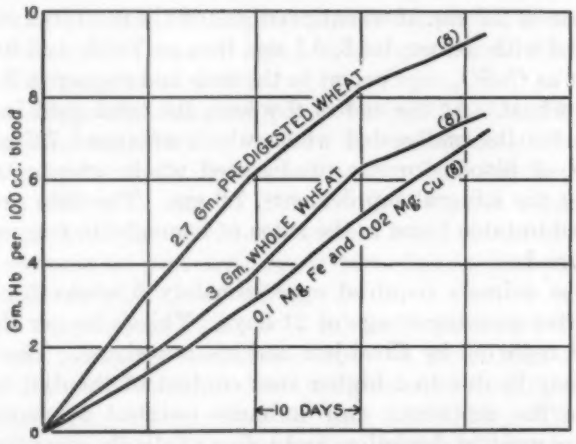

Fig. 1 Average weekly increases in hemoglobin from feeding the same amount of iron (0.1 mg.) and of copper (0.02 mg.) in form of whole wheat, predigested whole wheat, and iron and copper as $FeCl_3$ and $CuSO_4$. Figures in parentheses indicate the number of animals.

Failure to attain as good gains on the ash of whole wheat as on the original grain has been reported in a previous paper (Rose, et al., '32). Feeding the ash of 6 gm. of whole wheat resulted in 28 per cent less increase in hemoglobin in 6 weeks than occurred on feeding the whole wheat itself. In the present study the hemoglobin increase on the mineral supplements was only 8 per cent less than on the whole wheat but 21 per cent less than on the predigested wheat.

The findings with regard to gains in hemoglobin on 3 gm. of whole wheat are in good accord with our former report in which eleven animals, averaging hemoglobin 5.4 gm. and an age of 83 days at the end of the depletion period, showed an average gain of 6.7 gm. hemoglobin in 6 weeks. This confirms our view that in case of iron, as of protein, since it is not possible to deplete the body completely, any hemoglobin level low enough to afford opportunity for a considerable increase before reaching the full normal value is suitable for studies of the efficiency of foods in hemoglobin regeneration.

SUMMARY

Young rats depleted to hemoglobin levels averaging 3.3 gm. per 100 cc. of blood at 8 weeks of age were fed as supplements to fresh whole milk (1) whole wheat (3 gm.), yielding 0.1 mg. of iron and 0.02 mg. copper; (2) whole wheat (2.7 gm.) with all the starch dextrinized, furnishing the same amounts of iron and copper as (1); (3) 0.1 mg. of iron as $FeCl_3$ and 0.02 mg. copper as $CuSO_4$. Hemoglobin regeneration was best on the predigested wheat, next best on the natural wheat finely ground, and poorest on the mineral supplements, the gains in 6 weeks amounting to 9.6 and 7.6 and 7.0 gm. of hemoglobin per 100 cc. of blood respectively. The ease of digestion of the dextrinized wheat we believe to have been the factor causing the better hemoglobin regeneration on this than on the wheat finely ground but not predigested.

The gains in hemoglobin on 3 gm. of whole wheat in this series with animals depleted to an average hemoglobin level of 3.3 gm. of hemoglobin per 100 cc. of blood are in good agreement with those for animals in a previous series depleted to a level of 5.4 gm. As long as the level at depletion is sufficiently low to allow for considerable increase before reaching the normal hemoglobin level, we believe tests for hemoglobin regenerating values of foods will be reliable.

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PROTEIN UTILIZATION AS AFFECTED BY THE PRESENCE OF SMALL AMOUNTS OF BRAN OR ITS FIBER

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The concept of an adequate diet was exceedingly simple before the discovery of the vitamins and of the important roles in nutrition of a whole category of mineral elements. 'Balance' between the non-nitrogenous fat and carbohydrate on the one hand and the protein on the other, reduced to an isodynamic basis, was conceived to fulfill the conditions of good nutrition. With this idea dominant, the availability of the protein in any edible material became one of the chief criteria of its value as a food. Today we not only recognize many other substances in food besides protein as of prime nutritional significance, but also appreciate that the protein requirement of human beings is much lower than was formerly supposed. Hence the student of the American dietary is usually more concerned lest the supply of the vitamins and of certain mineral elements be inadequate than lest there be a real deficiency of protein. Foods must now be evaluated for the various nutrients which they may yield. Thus the tomato, once viewed askance by the physiologist because neither a good source of energy nor of protein, has come to be recommended as a staple in the diets of the poorest people because of its richness in vitamin C. Similarly there has been some change of emphasis with regard to the contributions made to the dietary by the cereal grains. While they remain of the greatest significance the world over as

cheap fuel for the human machine, they also constitute a most economical source of protein, iron and vitamin B. McCarrison ('28) has pointed out that in India the dependence of the mass of the population upon cereal food as a source of energy gives significance to differences in grains in other values besides calories. Rice even when not deprived of its bran coats is inferior to wheat in its power to support growth.

It is well known that the addition of cellulose to any ration lowers the coefficients of digestibility of its proteins, since cellulose calls out an increased secretion from the intestinal tract and the nitrogen in this secretion is not all reabsorbed but passes out in the feces. This effect was studied by Mendel and Fine ('12), who added 3 gm. of agar-agar and 7 gm. of bone ash to meat powder yielding 4.6 to 4.9 gm. nitrogen and found in dogs an increase in fecal nitrogen amounting to 60 per cent or more. Some increase of fecal nitrogen must, therefore, be expected when any cellulose is added to the diet, but for the moderate amounts of roughage which serve to keep the diet of the normal adult satisfactory as to laxation, this loss is not as great as many seem to think. Snyder ('01) in a series of three digestion experiments reported a difference of approximately 5 per cent between the coefficient of digestibility of the proteins of white flour and the proteins of the same flour to which had been added 14 per cent of finely ground bran to make it equivalent to graham flour. Newman and others ('12) in a study of white and whole wheat flour continued over 2 weeks found the average coefficient for the proteins of white flour 89.3 per cent while that for whole meal was 85.9 per cent, a difference in this relatively long experiment of only 3.4 per cent. Holmes ('19) summarizing various tests made by the U. S. Department of Agriculture, reports the average coefficient of digestibility for protein of white flour to be 88.1 per cent (thirty-one experiments) and for that of graham flour to be 76.9 per cent (twenty-four experiments), a difference of 11.8 per cent. Holmes ('19) investigated the digestibility of a diet in which the total bran averaged in one series 155 gm., finely ground, and in another 132 gm., coarsely ground. The bran was added to a ration

of potato, fruit, butter and sugar. The diet furnished on the average only 24 gm. of protein and the alimentary tract was so stimulated that the output of nitrogen occasionally exceeded the intake. The coefficient of digestibility for the protein of the diet was 37 per cent when fine bran was eaten and 35.8 per cent when coarse bran was substituted for fine. This was far too large an amount of bran to indicate what happens when only a small amount is added to the diet, or its influence when separated from the other portions of the grain and prepared for human consumption by thorough steaming and toasting.

From the commercial point of view the separation of endosperm, bran and germ has certain advantages. The perishable embryo, one of our richest and cheapest sources of vitamin B, can be suitably preserved and used to restore to the diet this factor which tends to be low when whole grains are exchanged for highly milled cereals. The bran not only furnishes roughage in a convenient form (Rose et al., '32) but is a good source of vitamin B (Rose, Vahlteich and Funnell, '32) and of iron (Rose, Vahlteich and MacLeod, '34) and contains proteins of excellent quality. According to Jones and Gersdorff ('25) 22 per cent of the nitrogen of the wheat kernel is in the bran. Murphy and Jones ('26) fed seventy young rats diets containing 70 per cent of bran, plus 12 per cent starch and 14 per cent butter fat, with suitable mineral and vitamin supplements and found the bran proteins efficient for growth for 6 weeks, gains being 1.83 gm. per gram of protein eaten.

It seemed worth while to find out what the effect upon the output of nitrogen of human beings would be when a small amount of prepared bran (approximately 1½ ounces) was added daily to a simple mixed diet, in comparison with the pure fiber from a similar portion of bran supplemented by crystalline vitamin B hydrochloride[1] to equal the amount of vitamin B present in the original bran.

Two healthy young women served as subjects. The experimental periods were each 9 days in length and a preliminary

[1] Kindly furnished by Mr. R. R. Williams.

period of 3 days on the experimental ration without collection of samples preceded each of the four periods. The basal diet consisted of milk, sugar, lactose, cornstarch, butter fat, cream, grape juice and apples. To this ration was added in the first period prepared bran, 45 gm. for one subject and 54 gm. for the other, the amount being adjusted so that the amount of nitrogen per kilo of body weight would be the same for each subject. Since in a study formerly made on animals (Rose et al., '32) it had been found that a small amount of bran added to a diet deficient in vitamin B did not induce the same laxative effect as when added to one in which vitamin B was liberal, the equivalent of the amount of vitamin B present in the bran was added in the periods in which pure fiber or no fiber was used, as an extra precaution to keep conditions as nearly uniform as possible. In the second period, pure bran fiber replaced the original bran. In the third period, in addition to the pure fiber, one subject received daily 76 units of vitamin B in the form of pure hydrochloride and the other subject 90 units daily. In the fourth period, the basal ration was taken with no addition except the vitamin B in the same amounts as in the third period. The starch, lactose and butter fat were made into wafers and baked till crisp. These served as a substitute for bread and were very palatable.

The total protein intake was limited to 0.5 gm. per kilo of body weight in order to keep the intake close to the minimum, and afford a better opportunity to observe the effect of the bran on the nitrogen balance. Also it served to keep the percentage of the total protein derived from bran fairly high, this amounting to 22 per cent throughout with the exception of the first 3 days of period I for subject M when it furnished 18.6 per cent.

All foods were weighed before ingestion and suitable samples were analyzed for total nitrogen by the Kjeldahl method. Urine and feces were collected daily, carmine being used as a marker for the feces. Analyses of urine and feces were made by 3-day periods. The food intake and the nitrogen content of each of the foods used are shown in table 1.

TABLE 1

Daily food intake and nitrogen content of diet

	SUBJECT F WEIGHT 58 KG.			SUBJECT M WEIGHT 68 KG.		
	Food	Intake	N in food	Food	Intake	N in food
		gm.	gm.		gm.	gm.
Period I	Milk	700	3.69	Milk	880[1]	4.54
	Cream	70		Cream	70	
Basal diet	Butter fat	40		Butter fat	20	
plus bran	Apple	250	0.04	Apple	250	0.04
	Lactose	75		Lactose	75	
	Bran	45	1.05	Bran	45[2]	1.05
	Sucrose	50		Sucrose	50	
	Cornstarch	60		Cornstarch	40	
	Grape juice	250		Grape juice	200	
	Total		4.78	Total		5.63
Period II	Milk	875	4.54	Milk	1050	5.53
	Cream	70		Cream	70	
Basal diet	Butter fat	40		Butter fat	30	
plus bran	Apple	250	0.04	Apple	250	0.04
fiber	Lactose	55		Lactose	20	
	Bran fiber	3.5	0.00	Bran fiber	3.5	0.00
	Sucrose	50		Sucrose	40	
	Cornstarch	60		Cornstarch	50	
	Grape juice	250		Grape juice	200	
	Total		4.58	Total		5.57
Period III	Milk	875	4.56	Milk	1050	5.39
	Cream	70		Cream	70	
Basal diet	Butter fat	40		Butter fat	40	
plus bran	Apple	250	0.04	Apple	250	0.04
fiber and	Lactose	55		Lactose	55	
vitamin B	Bran fiber	3.5	0.00	Bran fiber	3.5	0.00
	Sucrose	50		Sucrose	50	
	Cornstarch	60		Cornstarch	60	
	Vitamin B (75 units)			Vitamin B (90 units)		
	Total		4.60	Total		5.43
Period IV	Milk	875	4.50			
	Cream	70				
Basal diet	Butter fat	40				
plus vita-	Apple	250	0.04			
min B	Lactose	55				
	Sucrose	50				
	Cornstarch	60				
	Grape juice	250				
	Total		4.54			

[1] Changed after first 3 days to 845 gm.

[2] Changed after first 3 days to 54 gm.

For subject F, who was tall and thin, the allowance of 0.5 gm. of protein per kilo of body weight was slightly under requirement, as shown by a deficit of 0.92 gm. of nitrogen daily in the period when no bran or fiber was ingested and also by the fact that in each period the urinary nitrogen exceeded the nitrogen intake slightly. For subject M who had a heavier build and more body fat, the allowance slightly

TABLE 2

Daily nitrogen intake and output

	SUBJECT F						SUBJECT M					
	N in food	N in urine	N in feces	N output	N balance	Per cent N in feces	N in food	N in urine	N in feces	N output	N balance	Per cent N in feces
	gm.	gm.	gm.	gm.	gm.		gm.	gm.	gm.	gm.	gm.	
Period I Basal diet plus bran	4.78	4.98	1.29	6.27	— 1.49	27	5.67	4.99	1.27	6.26	— 0.59	22
Period II Basal diet plus bran fiber	4.59	4.80	0.92	5.72	— 1.13	20	5.57	5.31	0.85	6.16	— 0.59	15
Period III Basal diet plus bran fiber and vitamin B	4.61	4.70	0.86	5.56	— 0.95	19	5.43	4.32	0.99	5.31	+ 0.12	18
Period IV Basal diet plus vitamin B	4.54	4.73	0.73	5.46	— 0.92	16						

exceeded requirement, the urinary nitrogen falling short of the intake in all periods, and a slight positive balance of 0.12 gm. appearing in the period when fiber and vitamin B were taken. The daily balances are shown in table 2. Subject M was obliged to withdraw from the experiment at the close of the third period, on account of transfer of residence to another part of the country.

That the prepared bran was more laxative than its fiber, which had been finely subdivided by the vigorous treatment to which it had been subjected in purification, is clearly shown

TABLE 3

Weight of feces

	DAYS	SUBJECT F		SUBJECT M	
		Moist weight	Dry weight	Moist weight	Dry weight
		gm.	*gm.*	*gm.*	*gm.*
Period I Basal diet plus bran	1–3	380	122	430	91
	4–6	302	82	412	101
	7–9	370	91	505	115
	Total	1052	295	1347	307
Period II Basal diet plus bran fiber	1–3	227	62	229	58
	4–6	175	54	280	66
	7–9	230	55	232	55
	Total	622	171	741	179
Period III Basal diet plus bran fiber and vitamin B	1–3	209	60	297	74
	4–6	167	51	354	69
	7–9	274	52	298	65
	Total	650	163	949	208
Period IV Basal diet plus vitamin B	1–3	241	47		
	4–6	166	43		
	7–9	109	32		
	Total	516	122		

TABLE 4

Percentage excess of total weight of feces of bran period I over other periods (II, III, IV)

		SUBJECT F	SUBJECT M
		per cent	*per cent*
Excess in period I over period II	Moist feces	70	80
	Dry feces	70	70
Excess in period I over period III	Moist feces	61	42
	Dry feces	81	48
Excess in period I over period IV	Moist feces	104	
	Dry feces	142	

by comparing the fecal output in each period. The weights of feces, both moist and dry, are shown in table 3 and the percentage by which the weight of feces in the bran period exceeds that of each of the other periods in table 4. The

greatest difference in case of subject F is between the bran period and that period when no bran or fiber was taken, the weight of the moist feces in the former period being 104 per cent and of the dry feces 142 per cent of that of the latter period. The differences in the fiber periods (II and III) average 65 per cent for the dry feces. In case of subject M the differences are similar.

The effect on the proportion of the nitrogen intake excreted in the feces is shown in table 2. On closely comparable intakes, subject F excreted 27 per cent of the total intake in the feces and subject M 22 per cent when the diet was supplemented by the prepared bran, while on the two diets with the pure fiber, subject F excreted 19 and 20 per cent, and subject M, 15 and 18 per cent, respectively. The lowest percentage was, of course, in the period with neither bran nor fiber (subject F), 16 per cent. The periods with the added fiber but no bran protein show the mechanical effect of the fiber on the nitrogen excretion. Since vitamin B in the quantities added apparently made no difference, periods II and III may be averaged and the coefficient of digestibility of the protein estimated as 81 per cent for subject F and 83 per cent for subject M. The coefficient for the diet with the added bran is 73 per cent for subject F and 78 per cent for subject M. The difference must be assumed to represent loss of bran protein, and the coefficients of digestibility of this are therefore 45 per cent for subject F and 59 per cent for subject M.

The inclusion of the fiber, which had little direct laxative effect, as shown by the total fecal output in periods II and III reduced the coefficient of digestibility of the diet as a whole only about 3 per cent in case of subject F while the bran reduced it 11 per cent. Part of this probably represents unabsorbed bran proteins and part extra metabolic products due to the greater laxative effect of the bran.

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INFLUENCE OF THE RATION ON THE VITAMIN C CONTENT OF MILK[1]

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The subject of this paper is a controversial one. Early literature which has been quoted in most texts and reviews of the subject supports the conclusion that the ration has a marked influence on the vitamin C ascorbic acid content of milk. In a recent review (associates of L. A. Rogers, '35), it is stated, that "The evidence although conflicting leaves little doubt that the supply of vitamin C in the diet of a lactating animal affects the concentration of it in its milk."

As early as 1921, studies at this station (Hughes and others, '21) indicated little relation between the ration and the antiscorbutic properties of the milk. Since these findings were not in agreement with results reported elsewhere (Barnes and Hume, '19; Hart et al., '20; Dutcher et al., '20; Hess et al., '20), the work was repeated under carefully controlled conditions with essentially the same results (Hughes et al., '27).

Subsequent to the publication of this work, a more satisfactory method for assaying the vitamin C content of milk has been developed in other laboratories. By the use of these new methods, MacLeod ('27) and Schwartze, Murphy and Hann ('30) found the vitamin C potency of winter milk from ensilage fed cows to be higher than any heretofore reported. In the latter laboratory such milk was found to be almost

[1] Contribution no. 100, Department of Dairy Husbandry, no. 192, Department of Chemistry, and no. 63, Department of Veterinary Medicine.

if not actually as potent as the best summer milk obtained from cows on pasture. Kieferle and Zeiler ('26) also have reported that the milk of ensilage fed cows is richer in growth promoting and antiscorbutic agents than that of cows kept on the usual winter food. On the other hand, de Wildt and Brouwer ('30) could find no seasonal variation in the vitamin C content of cows' or goats' milk; while more recently Wijngaarden ('34), using titration methods, observed no noticeable change in the quantity of vitamin C after adding silage to the cows' ration.

In view of the lack of agreement in the results reported from different laboratories it seemed desirable to make further study of the problem using the more desirable procedure for biological assay and one of the more recently developed chemical tests.

EXPERIMENTAL

A. Biological tests

In 1932, a more extensive series of tests was run using the more approved technic. The basal ration fed the guinea pigs was as follows: skim milk powder, 30 per cent; rolled oats and wheat bran, 59 per cent; butterfat, 8 per cent; cod liver oil, 2 per cent; and salt 1 per cent.

The skim milk powder was heated for 4 hours at 110°C. with frequent stirring to insure as complete destruction of vitamin C as possible. This ration with the addition of orange juice has proved adequate for growth and the prevention of scurvy.

The guinea pigs used in this experiment were obtained from the animal genetics laboratory at this station. With ample numbers available care was taken to select only animals which proved satisfactory milk drinkers. Animals of approximately 300 gm. weight were chosen where possible.

Each guinea pig was maintained in a separate cage with wire bottom. The supply of the basal ration kept before each animal was replenished every 3 days and an accurate record of feed consumption maintained throughout the experiment. The animals were weighed at intervals of 3 days.

The milk was furnished in iron dishes. Morning and evening milk from the different lots of cows was fed within 2 hours of production. All milk was fed at the 40 cc. level with equal division between the two milkings. Furthermore, if any milk was unconsumed at the end of 2 hours, the pig was fed an equivalent amount of fresh milk by pipette. In this way total milk consumption was assured within a relatively short time after feeding. Since the guinea pig is not a heavy consumer of liquid, the results of earlier work may have been complicated by the lack of complete consumption.

The milk tested was from cows on several carefully controlled rations. Lot I had access to good brome and blue grass pasture and received sorghum silage and a grain mixture in addition. Lot II was fed a typical winter ration of alfalfa hay, sorghum silage, and a grain mixture; while lot III received the same ration except that silage was eliminated. Lots II and III were maintained in dry lot throughout the experiment. Lot IV consisted of a group of cows which had been in dry lot over a year, receiving a dry ration of prairie hay, beet pulp, corn, oats, and blood meal.

There were two cows in each lot, an Ayrshire and a Holstein, with the exception of lot IV, which consisted of two Holsteins. The average production and butterfat percentage of the different lots was fairly well balanced, though the butterfat percentage of lot II averaged nearly 1 per cent higher in the final 2 months.

The milk from the cows in each lot was mixed before each feeding and a composite sample used for feeding.

Close clinical observation was maintained on all guinea pigs throughout the experiment. At least two of the authors made daily examination of each animal for symptoms of scurvy. At death or on conclusion of the 90-day period autopsy findings were recorded on the basis of the scurvy score as developed by Sherman and his co-workers ('31).

Table 1 shows the results obtained from feeding the basal ration. It will be noted that first clinical signs of scurvy appeared in 16 to 17 days, while in the two lots tested, the

average days to death ranged from 31 to 36 days with a high scurvy score. This demonstrates the deficiency of the basal ration in vitamin C and the results obtained through its use are in accord with the findings of some of the more recent work in other laboratories.

Since several comparatively recent studies, previously mentioned, have attributed the relatively high vitamin C content of milk from stall fed cows to the silage which they were receiving, a test was made of the antiscorbutic properties of the silage which the cows were fed in this experiment, through direct feeding to a group of guinea pigs. Comparison of the results for average days to first signs of scurvy, average days

TABLE 1

Vitamin C content of silage

RATION	GUINEA PIGS (NO.)	AVERAGE INITIAL WEIGHT	AVERAGE MAXIMUM WEIGHT	AVERAGE FINAL WEIGHT	AVERAGE FIRST SIGNS SCURVY	AVERAGE DAYS TO DEATH	SCURVY SCORE
		gm.	*gm.*	*gm.*	*Days*		
Basal ('30)	10	308	328	216	17.0	31	41.7
Basal ('32)	9	326	353	229	15.9	36	56.5
Basal plus silage	10	273	323	207	16.8	35	38.8

to death, and average scurvy score for each group (table 1) would indicate that silage has little or no antiscorbutic properties as fed under the conditions of this experiment. While the scurvy score for the silage fed guinea pigs is below that of the basal groups, on the other hand, the average days to first signs of scurvy and the average days to death fall between the values for the basal groups. Ellis, Steenbock and Hart ('21) in earlier studies found silage to contain little if any vitamin C.

In table 2 the results are presented for the controls and the groups of guinea pigs receiving milk from the sources described. The controls which received the basal ration and 2 cc. of orange juice daily, made a more rapid growth with heavier food consumption than any of the milk fed lots.

Six guinea pigs in each lot completed the full 90 days. The others with one exception died at from 49 to 67 days.

Three of the eight animals fed silage milk showed clinical signs of scurvy. This, alone, would constitute evidence in favor of the pasture milk. This evidence is weakened, however, by the fact that only one animal showed clinical symptoms in each of lots III (no silage) and IV (dry ration). If one guinea pig in the silage milk group which died on the twenty-first day were eliminated, there would be no appreciable difference between the different lots in so far as the scurvy score is concerned.

While these results do not indicate any appreciable difference in the antiscorbutic properties of these milks, they quite

TABLE 2

Influence of ration on the vitamin C content of milk

RATION OF COWS	GUINEA PIGS (NO.)	AVERAGE INITIAL WEIGHT	AVERAGE MAXIMUM WEIGHT	AVERAGE FINAL WEIGHT	DRY FOOD CONSUMED	SCURVY SCORE
		gm.	*gm.*	*gm.*	*gm.*	
Controls—no milk	6	313	667	638	2112	0.0
Lot I pasture	9	347	582	555	1529	1.4
Lot II silage	8	327	540	532	1450	7.6
Lot III no silage	8	311	511	478	1472	1.3
Lot IV dry ration for 1 year	10	311	487	458	1173	0.49

obviously produced different effects on growth. It will be observed that the pasture milk group made the most substantial growth followed in order of decreasing growth by the silage, no silage, and dry ration lots. Elvehjem, Hart, Jackson and Weckel ('34) have demonstrated that mineralized pasture milk has a higher nutritive value when measured by rate of gain in rats, which require no vitamin C, than milk from cows on winter feed.

It seems reasonable, therefore, to conclude that the increased growth of the guinea pigs used in the present experiment is not the result of any increased vitamin C values in the milk. This in accord with the scurvy score and clinical findings as well as the results of the chemical analysis.

B. Chemical tests

Since the recently developed chemical tests for vitamin C provide a more satisfactory measure of this substance it was decided to continue the study using a chemical test.

It was possible to obtain, from feeding tests being carried on by the department of dairy husbandry, milk produced by cows on several controlled rations. At the beginning of the experiment ten cows were on the regular winter ration including silage. During the experiment six of these were turned out to young rye pasture. The other ten cows used were on dry winter ration without silage during the whole of this experiment. Evening milk was used for all tests and the titrations were completed within 2 hours after the time the milk was drawn.

As the titrating reagent 2-6 dichlorophenolindophenol was selected because it has been widely used and accepted and because it is claimed to be more nearly specific for vitamin C than other reagents such as methylene blue or ferricyanide mixtures (Tillmans et al., '32; Bessey and King, '33). This reagent, when used with milk serum that had been clarified with trichloracetic acid, was found to give definite reproducible results and to give complete recovery of commercial vitamin C that had been added to samples of milk. This commercial vitamin C was also used to standardize the dye for the other groups. Detailed results for the group of cows that received pasture during the latter part of the experiment are shown in table 3. These results are typical of those found in each group and only the average values are given.

The total numbers of samples tested, the average values of vitamin C in milligrams per liter and the standard deviations are shown in table 4.

The highest individual value observed was 37 mg. and the lowest 17 mg. The highest average for all results on one cow was 29.9 mg. per liter and the lowest similar average was 21.6. It will be seen that the average values for the three different types of milk are nearly identical, with the values for the pasture milk falling between the other two.

The significance of the close agreement between the average values for the different groups is increased by comparison with the large variation between individuals of a group and with the variation of a single individual from day to day.

These results confirm the biological findings in this laboratory. The failure of the ration to influence the vitamin C content of milk is in accord with the fact the cow is known to synthesize this vitamin (Thurston, Palmer and Eckles, '29).

TABLE 3

Influence of ration on the vitamin C content of milk
(vitamin C expressed as milligrams per liter)

COW NO.	MARCH						APRIL									MAY 1
	18	20	28	29	30	31	1	6	8	9	15	16	17	29	30	
1	26	30	24	28	28		30	28	26	21		31	24	26	26	
2	26	30	29	29	29	27	30	31	35	25						
3	26	36	24	28	28	24	28	33	35	26						
4	22	24	19	20	23	23	31	25	29	23						
5	22	27	21	25	23	24	24	26	29	23	29	24	22	26	24	22
6	22	30	25	25	26	24	26	30	29	23	22	26	29	29	24	24
Av.	24	29	24	26	26	25	28	29	30	24	26	27	25	27	25	23

Ration: alfalfa, silage and grain. Pasture after March 28th.

TABLE 4

Influence of the ration on the vitamin C content of milk

TYPE OF RATION	DRY RATION + SILAGE	PASTURE	DRY RATION, NO SILAGE
Number of samples tested	74	57	66
Vitamin C milligrams per liter	25.8	26.5	26.8
Standard deviation	4.0	3.2	3.9

SUMMARY

The influence of a number of carefully controlled rations on the vitamin C ascorbic acid content of milk was determined both by biological assay and chemical test.

Milk from cows receiving pasture was compared with milk from cows in dry lot receiving either silage or dry feed alone.

The results of these experiments indicate that the rations studied have no significant influence on the vitamin C content of milk.

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THE VARIATION IN THE MINERAL CONTENT OF VEGETABLES[1]

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INTRODUCTION

A balanced diet contains the five main types of food components: proteins, carbohydrates, fats, vitamins, and minerals. Deficiency in any of these food constituents causes material disturbances or diseased conditions.

The metabolic disturbances due to protein and vitamin deficiencies have been the subject of much study. Although disturbances due to the inadequate supply of minerals have been studied less than those due to other dietary deficiencies, nevertheless, several such disturbances have been recorded mostly within recent times.

The relation of goiter to an inadequate supply of iodine in the diet has long been recognized. The relation of secondary anaemia to an iron and copper deficiency is now well known (Elvehjem, '33). Functional disturbances due to a deficiency of manganese (Orent and McCollum, '31), and magnesium (Kruse, Orent and McCollum, '32) in the diet have been recorded recently by McCollum and his co-workers.

All these minerals are found in foods in relatively low concentrations or as in the case of magnesium, are required in very minute quantities to prevent functional disturbances. However, rickets exemplifies a nutritional mineral disturbance in which major ash constituents are involved, that is, calcium and phosphorus (McCollum, '22).

[1] Food Research Division contribution no. 230.

Klinke ('31) records abnormal mineral metabolism, including the major ash constituents, in a number of diseases, which are perhaps caused by the improper quantitative relation between the mineral constituents in the diet.

The major ash constituents also play an important role in determining the acid-base balance of foods—a field in animal nutrition which has been little explored since the work of Sherman and Gettler ('12), and Blatherwick ('14) but in which a revived interest is recorded in recent literature (Bell, '33; Kugelmas and King, '33).

Notwithstanding the importance of minerals in nutrition, the available data on the mineral composition of foods are incomplete and unreliable. The data found in Wolff's 'Aschen analysen' (1880) which has been for a long time the chief source of information on the mineral content of plant materials, are now considered unreliable and out of date in view of the cruder methods of analysis used at the time the tables were composed. Ragnar Berg ('25) published with reluctance his compilation of data on this subject, stating that in a general way the data were incorrect and misleading.

Earlier investigators apparently did not realize the significance of the variations in the mineral content of food plants. Their analyses were for the most part made on very few samples and very often on only one sample obtained at random. The object of the present work was to study the variation of the mineral composition of vegetables with variety, climate, soil, season, etc.[2] Leafy vegetables were studied first because of their increasing use in modern nutrition and because theoretically they are more subject to variation than fruits and seeds (Davidson, '33).

[2] When this work had been completed there appeared in Die Landwirtschaftlichen Versuchstationen (vol. 119, 1934, nos. 1–6 and vol. 121, 1934, nos. 5–6) two investigations dealing with the influence of fertilizers, variety and environment on the mineral composition of German forage crops.

EXPERIMENTAL

Materials used. The materials used in this investigation comprised six samples of spinach, six of kale, three of broccoli, two of lettuce and two of cauliflower.[3] In collecting the samples the following information was sought: a) variety of vegetable; b) where grown; c) type of soil; d) fertilizer treatment; e) was land irrigated?; f) analysis of irrigation water; g) kind of insecticide used; h) dates of planting and harvesting.

All these factors should theoretically affect the composition of the vegetables and might suggest an explanation of differences obtained in their analysis. Such information as was obtained from the growers regarding the materials used in this investigation is given in diagram 1.

Preparation of samples for analysis. An entire crate was used as one sample. The sound edible part of the material was weighed as soon as it was obtained, disregarding the weight of the foreign material which in first class vegetables is generally insignificant. In the case of spinach, however, several samples contained an undue amount of sand. In such cases the sand was collected during washing and its weight subtracted from that of the fresh sample.

The samples were washed and rinsed with distilled water and the excess of the adhering water was removed with towels or blotting paper. The material was then dried in a dryer (closed type, with fan running at 1300 to 1400 R.P.M.) at 60°C., re-weighed, ground in an iron mill and kept in Mason jars.

Methods of analysis. The methods used were mostly those of the Association of Official Agricultural Chemists ('30).

Potassium was determined as chloroplatinate; sodium by difference; calcium as oxalate titrated against N/10 potassium permanganate; magnesium gravimetrically; phosphorus volumetrically; sulphur gravimetrically, the materials having been ashed with magnesium nitrate; chlorine volumetrically;

[3] The study of these materials was intended as a part of a more extensive investigation, which, however, has been temporarily discontinued.

DIAGRAM 1

Description of vegetables

VEGETABLE [1]	VARIETY	LOCALITY	TYPE OF SOIL	FERTILIZER TREATMENT [2]	COMPOSITION OF IRRIGATION WATER
				lbs.	*p.p.m.*
Lettuce A	.....	Open market[3]	.....	.....	.....
Lettuce B	.....	Open market[3]	.....	.....	.....
Spinach A	.....	Open market[3]	.....	.....	.....
Spinach B	.....	Open market[3]	.....	.....	.....
Spinach C	Virginia Savoy	Diamond Springs, Va., Expt. Station Farm	Norfolk Loam	600 7–8–5 before planting 500 9–6–5 after thinning	Not irrigated
Spinach D	Virginia Savoy	Diamond Springs, Va., Expt. Station Farm	Norfolk Loam	Same as spinach C	Not irrigated
Spinach E	Virginia Savoy	Ocean View, Md.	Sassafras Sandy Loam	500 7–6–5 before planting 500 10–5–4 after thinning 500 $(NH_4)_2SO_4$ when half grown	Not irrigated
Spinach F	Virginia Savoy	Ocean View, Md.	Sassafras Sandy Loam	Same of spinach E	Not irrigated
Kale A	.....	Open market[3]	.....	.....	.....
Kale B	Dwarf Blue Curled Scotch	Diamond Springs, Va., Expt. Station Farm	Norfolk Loam	1000 5–8–5 before planting 200 $NaNO_3$ when half grown	Not irrigated
Kale C	Dwarf Blue Curled Scotch	Diamond Springs, Va., Expt. Station Farm	Norfolk Loam	Same as kale B	Not irrigated
Kale D	Dwarf Blue Curled Scotch	Ocean View, Md.	Sassafras Sandy Loam	1500 7–6–5 side dressed	Not irrigated
Kale E	Dwarf Blue Curled Scotch	Ocean View, Md.	Sassafras Sandy Loam	1500 7–6–5 side dressed	Not irrigated
Kale F	Tall Scotch Curled	Davis, Calif.	Yolo Silt Loam	Barnyard manure	Na 59, Ca 31, Mg 62, Cl 13, SO_4 22
Broccoli A	.....	Open market[3]	.....	.....	.....
Broccoli B	Italian Green Sprouting	College Park, Md.	Sandy Loam	12 (tons) manure 1000 6–6–5	Analysis not available
Broccoli C	Italian Green Sprouting	College Park, Md.	Sandy Loam	Same as broccoli B	Analysis not available
Cauliflower A	Early March	Salinas, Calif.	Very light gravel	Fertilized	Analysis not available
Cauliflower B	Early March	Salinas, Calif.	Very light gravel	Fertilized	Analysis not available

[1] Parts of vegetables analyzed: lettuce, tight heads (outer leaves and butts removed); spinach and kale, loose leaves, butts removed; broccoli, entire plant, except lower tough portion; cauliflower, flowering portion, butts removed.

[2] All applications are expressed in pounds per acre. The figures describing the fertilizers refer, in order, to the percentages of nitrogen as ammonia (NH_3), phosphorus as phosphoric acid (P_2O_5) and potassium as potash (K_2O).

[3] Washington, D. C.

and manganese colorimetrically by the periodate method (Davidson and Capen, '29).

Iron was determined by the thiocyanate method. The aliquots of the hydrochloric acid solutions were evaporated to dryness to render the silica insoluble. Skinner and Peterson ('28) state that this procedure interferes with the development of the thiocyanate color. Under the conditions of this work such interference was not observed. After evaporation to dryness the aliquots were taken up with 10 cc. of a 20 per cent solution of hydrochloric acid, oxidized with potassium permanganate until a permanent pinkish color was obtained and allowed to stand overnight. The next morning the solutions were always found decolorized, and the addition of permanganate was resumed until a permanent pinkish color was again obtained. Ten cubic centimeters of a 20 per cent solution of potassium thiocyanate were then added. The color appeared immediately and resisted fading for several hours. A Schreiner colorimeter was used in comparing the unknown with the standard solutions.[4]

Copper was determined by the Biazzo method (Elvehjem and Lindow, '29). Owing to the relatively high content of phosphorus, the copper was precipitated with hydrogen sulphide in an acid solution as suggested by Elvehjem and Lindow ('29). The precipitate was dissolved in nitric acid, and the solution evaporated nearly to dryness in order to avoid adding too much sodium hydroxide in neutralizing it, as the presence of an excess of sodium nitrate seemed to interfere with the development of the color. There was enough iron in the copper aliquots to develop an intense color with the thiocyanate reagent, but when allowed to stand this color faded and finally disappeared. In some cases the solutions were allowed to stand 2 days before the iron thiocyanate color disappeared. In such cases pyridine had to be added again in order to develop the full green color. When allowed

[4] The iron results, although apparently normal were not included in the tables being open to question because of the fact that the materials were ground in an iron mill.

to stand the solution had to be protected against evaporation as the chloroform used in this method slowly escaped through the supernatant layer of water solution. A Klett colorimeter was used.

The acid-base balance determinations were made according to a method developed in this laboratory which is based on the titration of the ash with corrections for sulphur and chlorine lost during ashing (Davidson and LeClerc, '35).

The moisture in the air-dry samples was determined by drying at 130°C. for 1 hour.

RESULTS

The results calculated to the water-free basis are given in table 1.

Two points are brought out clearly from the analysis of the five vegetables studied:

1. There is appreciable variation in the mineral content of the same vegetable when grown under different conditions.
2. Each vegetable studied has its own range of variation.

Several points of interest are brought out when the results of the analysis are correlated with the description of the samples given in diagram 1.

The spinach samples C, D, E, F belong to the same variety. C and D were taken from different points of a field in Virginia, E and F from different points of a field in Maryland. While there is no appreciable variation between the samples taken at different points of the same field, there is a wide variation between the samples taken from different fields, especially in K, Na, Ca, Cl and Mn. Particularly characteristic is the variation in the potassium and sodium content. The Virginia samples are higher in potassium and lower in sodium than those grown in Maryland. The smaller intake of potassium by the spinach plants from Maryland is compensated by a larger intake of sodium—a phenomenon well known in plant physiology. While the climatic conditions of the two fields are not very dissimilar, the soil types are different. The characteristic differences in the potassium and

ERRATA

In table 1 (p. 61) sulphur figures for spinach C, spinach D, spinach E, spinach F, kale B, kale C, kale D, kale E, broccoli B and broccoli C should be divided by two.

TABLE 1

Variations in mineral content and base balance of vegetables

VEGETABLES	MOISTURE IN FRESH MATERIAL	ON MOISTURE FREE BASIS										
		Ash	K	Na	Ca	Mg	P	S	Cl	Mn	Cu	Base balance
	Per cent	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*cc. N/10 alkali per gram of material*
Lettuce A	...	15.2	5.98	0.55	0.33	0.19	0.59	0.25	2.18	0.0069	.....	6.62
Lettuce B	...	13.2	6.27	...	0.45	0.27	0.98	0.29	1.20	0.0015	.....	....
Spinach A	...	19.1	7.75	0.73	0.69	0.33	1.01	0.66	1.94	0.0241	0.0028	....
Spinach B	...	20.8	6.80	...	0.57	0.58	0.79	0.66	1.19	0.0243	0.0033	13.11
Spinach C	94.0	20.8	7.43	0.70	1.26	0.50	0.96	0.96	0.52	0.0065	0.0073	21.87
Spinach D	95.3	21.4	6.94	0.99	1.49	0.47	0.79	0.95	0.58	0.0070	0.0066	23.32
Spinach E	96.0	20.5	4.14	2.37	1.83	0.34	0.81	0.93	0.66	0.0084	0.0045	20.29
Spinach F	93.7	19.7	4.35	1.62	2.11	0.37	0.92	1.07	0.93	0.0694	0.0043	18.12
Kale A	...	26.1[1]	2.90	0.97	1.37	0.32	0.25	0.64	0.71	0.0075	0.0031	....
Kale B	90.2	17.5	4.11	0.77	2.36	0.29	0.56	2.19	1.02	0.0137	0.0035	14.02
Kale C	92.7	15.4	3.49	0.70	2.07	0.33	0.64	2.27	0.96	0.0066	0.0039	10.72
Kale D	90.8	16.4	3.58	0.83	2.12	0.26	0.78	2.28	1.15	0.0060	0.0031	9.63
Kale E	91.2	15.1	4.37	0.83	1.83	0.24	0.68	2.20	1.20	0.0061	0.0056	8.03
Kale F	90.0	12.8	2.38	1.01	1.51	0.74	0.65	1.05	1.11	0.0011	0.0024	9.45
Broccoli A	...	24.7[1]	3.33	0.82	0.92	0.29	0.70	1.42	0.89	0.0032	.....	....
Broccoli B	87.8	12.6	3.60	0.40	1.48	0.22	0.70	2.44	0.78	0.0025	0.0033	3.79
Broccoli C	88.8	12.9	3.63	0.41	1.57	0.21	0.69	2.30	0.87	0.0021	0.0037	4.80
Cauliflower A	91.0	11.5	3.71	0.64	0.20	0.24	0.82	1.13	0.47	0.0009	0.0045	3.19
Cauliflower B	90.6	10.9	3.45	0.60	0.22	0.25	0.84	1.01	0.50	0.0007	0.0047	2.63

[1] Sample was dried and ground without having been previously washed.

sodium content of the samples from the two fields might be due to differences in soil. However, on consulting diagram 1, it is found that these two fields, besides being located on different soil types, have also received different fertilizer treatment. The Maryland field received an application of ammonium sulphate when the plants were half grown. It has been reported elsewhere (Davidson, '33), that a relatively large application of sodium nitrate, which is physiologically an alkaline fertilizer, caused an increased potassium intake by wheat plants. It is therefore possible that in a similar way ammonium sulphate, which is physiologically an acid fertilizer, caused a decreased potassium intake by the spinach plants.

The kale samples B and C were grown in the same field in the same locality as the spinach samples C and D, and the samples D and E in the same field and in the same locality as the spinach samples E and F. There is less variation in the mineral composition between the kale samples grown on different fields than between the corresponding spinach samples. The peculiar differences in the potassium and sodium content in the four spinach samples is not observed in the corresponding kale samples. This would seem to lend support to the possibility that the differences in the potassium and sodium contents of the spinach samples were caused by differences in fertilizer treatment rather than by differences in soil type.

The failure of the sodium nitrate application to cause an increased potassium content in the kale samples B and C as compared with samples D and E may be explained by the fact that this application was not sufficiently large to produce the effect referred to above (Davidson, '33). Furthermore, the two sets of samples cannot be well compared owing to the fact that the kale samples D and E received a much larger application of nitrogen in the complete fertilizer than B and C and there is no information as to how much of this nitrogen was in the nitrate form.

The kale samples B, C, D, E, differ quite markedly from samples A and F, especially in potassium, calcium and sulphur content.

The kale sample F was grown under irrigation. The irrigation water was appreciably richer in sodium and magnesium than in the other minerals. This sample has a higher sodium and magnesium content than the other five kale samples.

The broccoli samples B and C were taken quite close to each other. They differ but little from each other but differ appreciably from sample A, which was obtained from the open market.

The cauliflowers, taken at different parts of the same field, differ but little in composition.

Kale sample A and broccoli sample A have an exceptionally high ash content while the individual ash constituents are not correspondingly high. These samples having been obtained by another laboratory for a special purpose were dried and ground without having been previously washed, thus the high ash content may have been caused by the adhering soil particles.

In table 2 are given the extremes of variation of the ash, and of the individual ash constituents as well as those of the base balance and moisture content for each of the five vegetables studied. In a special column for each vegetable are given the percentages of extreme variation obtained by dividing the higher extreme by the lower and multiplying the result by 100, i.e., by considering the minimum as 100 per cent.

Spinach and kale varied much more than the other vegetables. However, this may be due to the fact that a larger number of samples of these vegetables than of the others was examined.

The range of variation of the total ash content is relatively small and not correlated with the range of variation of the individual ash constituents. This may be explained by the fact that a low content of one ash constituent is often compensated by a high content of another constituent. Correlation between the ash and its individual constituents is further

TABLE 2

Range of variation in composition of vegetables

CONSTITUENTS	LETTUCE TWO SAMPLES		SPINACH SIX SAMPLES		KALE SIX SAMPLES		BROCCOLI THREE SAMPLES		CAULIFLOWER TWO SAMPLES	
	Per cent	*Per cent variation*	*Per cent*	*Per cent variation*	*Per cent*	*Per cent variation*	*Per cent*	*Per cent variation*	*Per cent*	*Per cent variation*
Ash	13.2 –15.2	115	19.1 –21.4	112	12.8 –17.5	137	12.6 –12.9	102	10.9 –11.5	106
Potassium	5.98 – 6.27	105	4.14 – 7.75	187	2.38 – 4.37	184	3.33 – 3.63	109	3.45 – 3.71	108
Sodium	.....	...	0.70 – 2.37	339	0.70 – 1.01	144	0.40 – 0.82	205	0.60 – 0.64	107
Calcium	0.33 – 0.45	136	0.57 – 2.11	370	1.37 – 2.36	172	0.92 – 1.57	171	0.20 – 0.22	110
Magnesium	0.19 – 0.27	142	0.33 – 0.58	176	0.24 – 0.74	308	0.21 – 0.29	133	0.24 – 0.25	104
Phosphorus	0.59 – 0.98	166	0.79 – 1.01	126	0.25 – 0.78	312	0.69 – 0.70	101	0.82 – 0.84	102
Sulphur	0.25 – 0.29	116	0.66 – 1.07	162	0.64 – 2.28	356	1.42 – 2.44	172	1.01 – 1.13	112
Chlorine	1.20 – 2.18	182	0.52 – 1.94	392	0.71 – 1.20	169	0.78 – 0.89	114	0.47 – 0.50	106
Manganese	0.0015– 0.0069	460	0.0065– 0.0694	1068	0.0011– 0.0137	1245	0.0021– 0.0032	152	0.0007– 0.0009	129
Copper	.....	...	0.0028– 0.0073	261	0.0024– 0.0056	233	0.0033– 0.0037	112	0.0042– 0.0045	107
Base balance[1]	.....	...	13.11 –23.32	178	8.03 –14.02	175	3.79 – 4.80	127	2.63 – 3.19	117
Moisture	.....	...	93.7 –96.0	102	90.0 –92.7	103	87.8 –88.8	101	90.6 –91.0	104

[1] Cubic centimeters of N/10 alkali per gram of dry material.

obscured by the fact that two major (referring to quantity) ash constituents, the silica and carbon dioxide, were not determined.

The range of variation of potassium and phosphorus is in general smaller than that of the other major elements, possibly because vegetables are usually grown with complete fertilizer, which always contains these two elements.

The range of variation of the base balances is significant. In the study of the effect of the acid-base balance of foods on animal metabolism a variation of 178 per cent, as in the case of the spinach samples, would preclude any possibility of correlating the acidity and ammonia content of the urine with acid-base balance values found in the literature. For close correlations the acid-base balance values will have to be determined in each case.

The analytical data on the whole bear out the main thesis which it was intended to prove by this investigation, namely, that the mineral content of vegetables must be studied with the object of determining the ranges of variation rather than single values.

SUMMARY

Several samples of each of the vegetables, spinach, kale, lettuce, broccoli and cauliflower were analyzed for total ash, potassium, sodium, calcium, magnesium, phosphorus, chlorine, iron, manganese, copper and the base balance. The conditions under which most of the vegetables were grown are described.

The results show that—

1. There is considerable variation in the mineral content and base balance values of each vegetable studied.
2. Each vegetable studied has its own range of variation.
3. In several instances the mineral composition of the vegetables seems to be correlated with the fertilizer treatment or with the chemical composition of the irrigation water.

The results also seem to lead to the general conclusion that the mineral content of vegetables should be studied with the object of determining ranges of variation rather than fixed values.

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STUDIES ON VITAMIN G(B_2) AND ITS RELATION TO CANINE BLACK TONGUE [1]

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TWO FIGURES

(Received for publication September 16, 1935)

In a previous paper (Elvehjem and Koehn, '35) we reported that the symptoms of pellagra produced in chicks by feeding a heat-treated natural grain ration could not be cured by supplementing the basal ration with flavin preparations from liver or liver extract, but that the pellagra was readily prevented or cured when the filtrate from which the flavin had been separated was used as the supplement. It was suggested therefore that the term vitamin G(B_2) be retained for the factor remaining in the filtrate which cured the pellagra, and that the classification of the flavins be postponed until more was known about their biological activity.

Shortly after the appearance of our work, three independent groups of workers presented data obtained from studies on rats which verified our experimental results. Chick, Copping, and Edgar ('35) found that the addition of hepatoflavin or lactoflavin to a basal diet deprived of vitamin G restored the growth in rats to a small extent but was without effect on the characteristic florid dermatitis. A supplementary substance obtained from yeast extract had no effect in restoring growth but did have a slight curative effect on the dermatitis. When the flavin and supplementary material from yeast were given

[1] Published with the permission of the director of the Wisconsin Agricultural Experiment Station.

[2] Supported in part by a grant from the research funds of the university.

together, growth and cure of the dermatitis resulted. György ('35 a) found lactoflavin to have no connection with the pellagra-like dermatitis in 211 rats. He also found a combination of the flavin and a supplementary substance necessary for normal performance, and that the distribution of the two substances in nature varies to a considerable extent. Harris ('35) also found flavins to have no antipellagric action. All these workers discuss the possible nomenclature for the factors in question and suggest that vitamin G should be used for the complex and that new names should be given to the several constituents. However, these workers are not in agreement concerning the designation of the individual factors.

The final decision concerning this question must be left to a competent committee, but since the term vitamin G(B_2) has always been associated with the P-P factor of Goldberger, a question of prime importance is the relation of these factors to human pellagra.

At present there is no definite evidence in the literature to demonstrate that the pellagra obtained in chicks is identical with human pellagra. This is also true in the case of rats. Although Goldberger and Lillie ('26) described the production of specific symptoms in rats peculiar to vitamin G low diets which they conclude with some reservation to be the analogue of pellagra in man, the vitamin G low diets used in most laboratories today are not only different from the one described by Goldberger and Lillie, but the typical symptoms are rarely seen in many laboratories.

Eventually the different factors which have been separated must be tested on uncomplicated cases of human pellagra. However, since clinical studies of this nature are difficult to conduct, and since most authorities today agree that black tongue in dogs is identical with human pellagra both from symptomatic and etiological standpoint (Wheeler, '30), we were interested in repeating our work on the separation of vitamin G and flavins when dogs were used as the experimental animal.

The work to be reported in this paper was done on liver extract[3] similar to that used in the experiments with chicks. Goldberger and Sebrell ('30) have shown that liver extract no. 343 given by mouth will cure black tongue in dogs. Rhoads and Miller ('33) prevented black tongue in dogs by feeding 4 gm. daily of liver extract. Ramsdell and Magness ('33) and Ruffin and Smith ('34) have found that large amounts of liver extract by mouth are efficacious in treating human pellagra, while Spies ('34) has demonstrated that either the intravenous or the intramuscular administration of large amounts of potent liver extract causes a remission of the oral manifestations of pellagra.

EXPERIMENTAL

The basal ration was essentially diet no. 323 of Goldberger, Wheeler, Rogers, and Sebrell ('30) except that the cow peas were omitted, and the calcium supplied as calcium carbonate and calcium phosphate. It had the following composition, yellow corn 72, casein (heated 120°C. 24 hours) 18, cotton seed oil 5, cod liver oil 2, calcium carbonate 1, calcium phosphate 1, and NaCl 1, and will be referred to in this paper as ration 250. The dry mixture was prepared fresh each week and for feeding was suspended in hot water, cooked for a few minutes, and fed ad libitum twice daily. Two litters of dogs were used. The dogs in litter no. 1 (Collie breed) were weaned at 4 weeks of age, placed on ration 250 and commercial dog food for 5 weeks and restricted to the basal ration alone at 9 weeks of age. The dogs in litter no. 2 (rat terrier breed) were weaned at 4 weeks of age and started on the basal ration when 6 weeks of age. All dogs but one developed typical black tongue in 4 to 10 weeks. One animal in litter no. 2 for some unknown reason failed to develop the typical symptoms shown by his litter mates. The growth records for two dogs from litter no. 1, which were allowed to die of black tongue are shown in figure 1.

[3] We are indebted to Dr. H. W. Rhodehamel of Eli Lilly and Company, Dr. C. Nielson of the Abbott Laboratories, and Dr. David Klein of the Wilson Laboratories, who kindly supplied us with liver extract.

The symptoms observed were anorexia, loss of weight, lesions and hemorrhage of the tongue and labial and buccal mucous membranes of the mouth, excessive secretions from the eyes, vomiting, bloody diarrhea and general emaciation.

Dogs receiving supplements were placed in separate cages and given the material to be tested in a small amount of the basal ration each morning. After this material was consumed, the basal ration alone was supplied ad libitum. In cases of

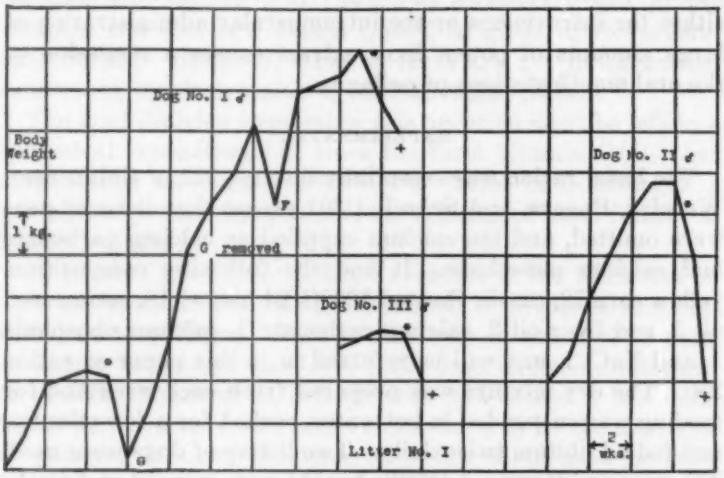

Fig. 1 Growth records of dogs receiving the basal ration and basal ration plus supplement. G denotes the administration of the vitamin G supplement. F denotes the administration of the flavin supplement. * appearance of black tongue.

severe black tongue, where the dog completely refused to eat, the supplement was given orally by pipette.

The liver extract was separated into the two fractions in the following manner: 100-gm. portions of liver extract were dissolved in 200 cc. of distilled water, and 1 liter of ethyl alcohol and 1.2 liters of diethyl ether were added with constant stirring. The solution was filtered, the precipitate washed with 250 cc. of a mixture of alcohol, ether, and water, and the washings combined with the filtrate. The solution was concentrated in vacuo to a volume of approximately 50 cc., filtered

and diluted to 1 liter with distilled water. Sixty cubic centimeters of concentrated HCl and 35 gm. of fuller's earth were added and the mixture shaken for 1 hour in a mechanical shaker. The fuller's earth was allowed to settle out over night, the supernatant liquid siphoned off, and the earth drained on quantitative filter paper. It was then washed by suspending in two 1-liter portions of distilled water.

The flavin was eluted by suspending the fuller's earth in a mixture of 250 cc. of water, 65 cc. of pyridine, and 65 cc. of methyl alcohol, and shaking for 1 hour. The fuller's earth was removed by centrifuging, the liquid concentrated in vacuo to 10 cc. to remove the pyridine, methyl alcohol, and colloidal fuller's earth, and diluted to 200 cc. In the above procedure, the operations were carried out in such a manner as to exclude exposure of the solutions to light.

The filtrate remaining after the flavins had been removed with fuller's earth was neutralized with NaOH and concentrated in vacuo to 50 cc. Three hundred cubic centimeters of 95 per cent ethyl alcohol were added and the precipitated NaCl was filtered off. The solution was concentrated to 10 cc. and diluted with water to 100 cc.

These supplements were fed at a level equivalent to 5 gm. of liver extract daily. This level was found by Goldberger and Sebrell ('30) to be just sufficient to prevent black tongue.

Dog no. 1 of litter no. 1 stopped growing at the end of the fourth week and developed severe black tongue at the end of the fifth week. At the end of the sixth week the vitamin G fraction was administered whereupon the symptoms disappeared and a rapid gain in weight followed. This supplement was given for 3 weeks and then removed. The dog continued growing for 3 weeks and then declined in weight. The flavin fraction was then used to supplement the ration. For 3 weeks the dog gained weight, then developed severe black tongue and died within 2 weeks (fig. 1).

In litter no. 2, dog no. 4 began losing weight at the end of the eighth week. The flavin fraction was administered whereupon severe black tongue developed with a rapid loss in

weight for 3½ weeks. The flavin supplement was then replaced by the vitamin G fraction. A very rapid increase in weight occurred with immediate disappearance of black tongue symptoms (fig. 2). After receiving this supplement for 5 weeks, the dog was killed and autopsied. Autopsy showed all organs to be normal. There were indications that there had been lesions in the lining of the small intestines but these lesions had been completely healed at the time of autopsy.

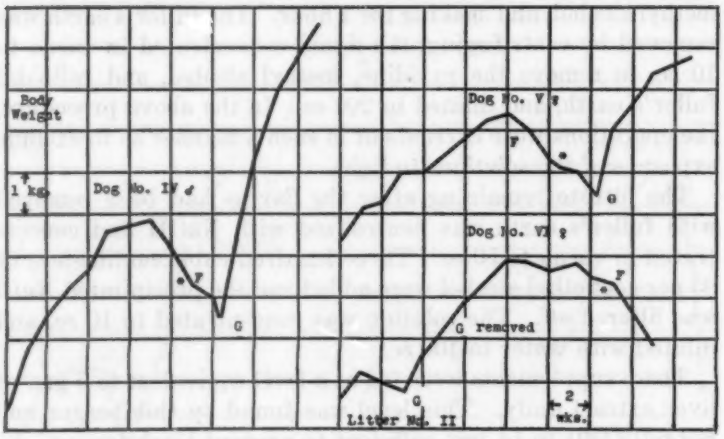

Fig. 2 Growth records of dogs receiving basal ration plus flavin fraction or vitamin G fraction. G denotes the administration of the vitamin G supplement. F denotes the administration of the flavin supplement. * appearance of black tongue.

Dog no. 5 stopped growing at the end of the eighth week and started losing weight. The ration was supplemented by the flavin fraction whereupon black tongue developed and the loss of weight continued. After receiving the flavin supplement for three weeks, the dog exhibited very severe symptoms of black tongue and extreme emaciation. The flavin fraction was withdrawn and replaced by the vitamin G fraction. Immediate disappearance of the symptoms of black tongue and resumption of growth occurred (fig. 2). The dog was killed and autopsied after receiving the vitamin G fraction for 5 weeks.

Autopsy as before revealed healed lesions of the intestinal tract.

Dog no. 6 lost weight after the end of the first week. At the end of the third week the vitamin G fraction was administered. Immediate resumption of grow resulted. After 3 weeks the supplement was removed and the dog continued growing for several weeks. At 13 weeks black tongue developed and the flavin supplement was given. Rapid loss in weight and extreme emaciation resulted. Autopsy revealed severe lesions and hemorrhages in the gastro-intestinal tract.

DISCUSSION

The fact that flavin prepared from liver extract has proven ineffective in the cure of canine black tongue, which has been shown to be analogous to human pellagra, gives additional and more conclusive evidence that flavins are not the antipellagric vitamin. Further support is given to the suggestion that vitamin G be retained for the flavin-free fraction active in the prevention of pellagra. Whether the fraction which has been found active for both chicks and dogs contains only one specific active substance cannot be answered at this time. Our work on the purification of the antipellagric factor indicates that there is only one substance concerned with this deficiency disease.

It is also difficult to determine whether the factor we have been concerned with is identical with the active principle studied by Chick ('35), György ('35 a), and Harris ('35), which they found in a yeast preparation. György suggested that the pellagra preventing substance in yeast as tested on rats be designated vitamin B_6. In this connection it is well to mention that György ('35 b) found whole liver to be a rich source of this factor. However, in a later publication Birch and György ('35) state that from a study of the distribution of B_6 and the flavin in cereal products, it appears highly probable that neither of these substances is identical with the human P-P factor of Goldberger. György ('35 c) also found the pellagra-preventing substance as studied in rats (vitamin

B_6) to be destroyed by visible light. We have been unable to demonstrate any decrease in the potency of concentrates active in the prevention of pellagra in chicks when exposed to either visible or ultra violet light. Reference is made to the work of Hogan and Richardson ('34) who showed that the pellagra-like dermatitis in rats was produced more readily when certain components of the diet were irradiated. György repeated this work and concluded therefrom that the supplementary factor (vitamin B_6) will cure the dermatitis produced by Hogan's method. However, in a recent paper Hogan and Richardson ('35) conclude that the dermatitis in their rats is cured by an ether extract of wheat germ. Thus there are probably several types of dermatitis and several factors concerned in the prevention of the development of each of these syndromes. Very recently von Euler and Malmberg ('35) report definite skin changes in rats on their basal diet plus 10 γ flavin and a flavin-free yeast preparation. Highly purified rations of casein, sugar, or starch and salts must be deficient in a number of factors, and different symptoms are encountered depending upon the purification of the casein and starch and the nature of the supplements used as sources of the known vitamins. If we are to test for the true antipellagric vitamin, all factors but the one in question must be present. This means that flavins must be present in the basal diet as well as vitamins A, D, B, etc. and not included as part of the vitamin G complex. If we accept the terminology of the committee on vitamin B nomenclature of the American Society of Biological Chemistry ('29) that G(B_2) be used to denote the more heat-stable, water-soluble, dietary factor called the P-P factor by Goldberger and associates, and which also has to do with maintenance and growth, then it seems most logical to call the factor which remains after the flavins are removed on fuller's earth and which has been shown to be active in the prevention of pellagra-like symptoms in chicks and black tongue in dogs, vitamin G(B_2).

SUMMARY

1. Typical symptoms of black tongue were produced in dogs by feeding a modified Goldberger ration.

2. Preparations of hepatoflavin prepared from liver extract were found to be completely inactive in the prevention or cure of black tongue.

3. Fractions prepared from liver extract which were rich in vitamin G(B_2) and from which the hepatoflavin had been removed through adsorption on fuller's earth were very active in the cure of black tongue.

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EFFECTS OF EXCESSIVE INGESTION OF SODIUM AND POTASSIUM SALTS ON CARBOHYDRATE METABOLISM AND BLOOD PRESSURE IN DIABETIC CHILDREN [1]

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NINE FIGURES

(Received for publication September 11, 1935)

In a preliminary communication (Thompson and McQuarrie, '34), the finding of an apparently significant influence of excessive salt ingestion on arterial blood pressure and carbohydrate metabolism was reported. The present paper records the results from a more extensive investigation of the subject.

Our interest in the question of a possible interrelationship between salt ingestion and carbohydrate metabolism was first aroused in 1930, when one of our younger diabetic patients, suffering from an abnormal craving for salt, appeared to show some freakish variations in her ability to utilize carbohydrate according to variations in the amounts of sodium chloride which she was allowed to consume. Lack of cooperation on the part of the patient and her premature discharge from the hospital frustrated our first attempts to investigate the problem. A second opportunity presented itself, however, in the autumn of 1933, when one of our clinic patients, a 15-year-old boy with moderately severe diabetes manifested a similar but even more marked craving for salt.

Obviously the first question presenting itself was that regarding the factors responsible for this patient's requiring

[1] Aided by a grant from the Medical Graduate Research Fund of the University of Minnesota.

between 80 and 90 gm. of table salt daily to satisfy his abnormal craving. Since he had been on a relatively high vegetable diet (high in K) for a long time, had partially depleted his reserve of base as a result of ketosis and had vomited an undetermined number of times just before being admitted to the hospital (thereby losing a considerable amount of chloride), this symptom was first tentatively explained on the simple basis of temporarily physiological need for both Na and Cl. That this explanation was entirely inadequate soon became apparent, however, when the craving was observed to continue only slightly abated long after all evidence of ketosis had disappeared and after a simplified diet low in potassium had been substituted for the ordinary diabetic diet. The unexpected discovery, that this patient's blood pressure was elevated temporarily and his glycosuria was decreased as a result of the extra salt ingestion, impressed us as being of sufficient interest to warrant immediate investigation. It appeared probable too that the factors involved in these reactions might incidentally provide an answer to our query concerning the symptom of salt craving.

Following confirmation of our original observation regarding the effects of high NaCl intake, a number of pertinent new questions presented themselves. Among these were the following: 1) Are the observed reactions peculiar to diabetic subjects suffering from an abnormal craving for salt or do other diabetics and even normal persons behave similarly under the same conditions? 2) Are the observed effects due specifically to the Na or the Cl ion alone or to the two combined? 3) Are the two effects (i.e., decrease in glycosuria and increase in blood pressure) mutually dependent or is it possible to obtain one without the other? 4) May the effect on blood pressure be due to increased tonus in the peripheral arterioles, to effects on the kidneys or merely to the mechanical effect of increased blood volume? 5) Is the diminution in glycosuria due merely to retention of sugar with retained water or is it due to improved ability to store or oxidize glucose? 6) If either of the latter, what is the nature of the

mechanism involved? 7) What nervous or endocrine functions, if any, are involved? 8) Has a high salt intake any influence on the metabolism of protein and fat?

METHODS AND MATERIALS

Repeated studies were carried out on each of four diabetic patients and on one normal subject, all in the age group between 13 and 15 years. Not more than two patients were under observation at one time. At such time they were kept in a small metabolism ward constantly under the supervision of special nurses, who weighed them every 12 hours, collected the urine and feces in special containers and made certain that diets and medications were taken precisely as ordered. Urine was collected quantitatively in 6- or 12-hour periods. The feces for different periods were marked by carmine according to standard technic. Systolic and diastolic blood pressure measurements were made by means of the same mercury sphygmomanometer before meals and after the patient had been in the recumbent position for at least ½ hour. Measurements were frequently checked independently by two observers. The averages of these readings were charted each day. The room temperature was held between 68° and 74°F. and the relative humidity between 40 and 60 per cent to prevent sweating or chilling. The body weight was determined every 12 or 24 hours immediately after the bladder was emptied and before meals or water were given.

The daily diet, which was constant for each patient throughout the entire experimental period, was divided into four identical meals given at 6-hour intervals. For the sake of uniformity and accuracy, it was made up from the following foods: powdered whole milk, egg yolk, egg white, cane sugar, water, unsalted butter, clear lemon juice and a small amount of white bread. The mineral constituents of a typical basic diet, containing protein 64, fat 96, and carbohydrate 132 gm., were as follows; Na, 1.03; K, 1.34; Ca, 0.68; Mg, 0.16; Cl, 1.83; P, 0.93 and S, 0.76 gm. It is seen that this is relatively low in sodium, potassium and choride. The amounts of protein,

fat and carbohydrate were adjusted to meet each patient's caloric and nitrogen requirements. The total water intake, based on the maximum amount taken during periods of high NaCl ingestion, was the same throughout individual experiments. The insulin dosages, in those cases on insulin therapy, were given at 6-hour intervals and were adjusted at levels which allowed some degree of glycosuria in every period of the day. When extra salt was added, it was given in the form of weak solutions and in gelatin capsules, the total for the day being administered along with the diet in four equal parts. Only chemically pure salts were used.

The urinary glucose was determined on the fresh samples in 6- or 12-hour periods by the method of Benedict ('11). The total nitrogen and the various mineral constituents were determined on 24-hour samples. The chemical methods used for the analyses of urine and feces were as follows: total nitrogen, Kjeldahl (1883); sodium, Salit ('32); potassium, Shohl and Bennett ('28); chloride, Volhardt, as modified by Harvey ('10). For blood analyses the methods were as follows: glucose, Folin and Wu ('20); sodium, Butler and Tuthill ('31); potassium, Shohl and Bennett ('28); chloride, Wilson and Ball ('28). The blood volume was determined by the dye method of Keith, Rowntree and Geraghty ('15) as modified by Hooper, Smith, Belt and Whipple ('20). Fasting respiratory quotients were kindly determined for us by Mr. A. J. Beber in the laboratory of Dr. George Burr.

EXPERIMENTAL RESULTS

For the sake of brevity, representative data from the various experiments are here presented by means of one short table and a series of graphic charts.

Table 1 contains the data from a single study on the various effects produced by the ingestion of 60 gm. of NaCl daily for a period of 1 week in the case of L.R., the 15-year-old boy who craved salt. It will be seen that the degree of glycosuria fell far below the control level soon after the extra salt was introduced and remained comparatively low throughout the experi-

mental period. That this change was due to the salt is further evidenced by the fact that glucose output again gradually rose to its previous level during the 4-day-post-saline control period. Approximately 27 gm. of NaCl was retained during the first day of the experimental period, but thereafter the output was practically equal to the intake. The gain of 2.5 kg. in body weight, which accompanied the retention of salt, undoubtedly represented chiefly retention of water. At an assumed Na concentration of 152 milli-equivalents per liter, that

TABLE 1

The effects of ingesting 60 gm. of NaCl daily on L.R. age 15 years. Severe diabetic. Diet contained protein 64, fat 132 and carbohydrate 168 gm. Insulin 32 units daily. No ketosis

DAY	INTAKE (GRAMS)				OUTPUT (GRAMS)					WEIGHT (KILOGRAMS)	BLOOD PRESSURE
	Na	Cl	K	N	Na	Cl	K	N	Glucose		
1	1.57	2.43	1.48	10.36	1.86	2.27	0.86	12.40	73	38.3	116/80
2	25.16	38.84	1.48	10.36	14.26	13.38	0.86	11.10	33	38.2	116/76
3	25.16	38.84	1.48	10.36	24.17	42.28	0.86	7.87	13	40.8	140/88
4	25.16	38.84	1.48	10.36	23.55	38.16	1.40	9.23	14	40.3	150/98
5	25.16	38.84	1.48	10.36	26.56	43.06	0.93	9.63	33	40.3	174/110
6	25.16	38.84	1.48	10.36	24.38	33.58	1.56	9.67	31	40.2	166/108
7	25.16	38.84	1.48	10.36	24.83	40.36	1.40	9.78	26	40.4	164/112
8	25.16	38.84	1.48	10.36	27.28	41.85	1.21	10.87	26	40.4	176/118
9	1.57	2.43	1.48	10.36	4.99	7.88	1.44	8.78	30	40.4	164/98
10	1.57	2.43	1.48	10.36	1.86	2.41	0.82	8.91	49	39.2	130/88
11	1.57	2.43	1.48	10.36	2.57	3.80	1.13	9.47	57	39.1	112/80
12	1.57	2.43	1.48	10.36	1.75	2.24	1.05	10.87	72	38.7	106/74

ordinarily assigned to extracellular body water, the retained water would account for but 22 gm. of the NaCl not excreted by way of the urine and feces, leaving 5 gm. unaccounted for. Since visible sweating did not occur, it is obvious that the concentration of NaCl in the body fluids was increased or that the extra salt was stored in some other form than in aqueous solution. Actual determinations of the Na and Cl in the blood serum a few days before and again a few days after the ingestion of the extra 60 gm. of NaCl per day were as follows: before, Na 303 mg. and Cl 281 mg. per 100 cc. of serum; after:

Na 342 mg. and Cl 342 mg. per 100 cc. The fasting blood sugar values for the corresponding days were 290 and 131 mg. per 100 cc. respectively. Increased concentration of salt in the body fluids of this particular patient, therefore, account in all probability for most of the extra NaCl retained. A comparison of the average per diem excretion of potassium for the experimental period with that for the fore-period and after-period shows an increased output of this element during the period of high NaCl intake.

The total nitrogen excretion was definitely decreased during the periods of high salt ingestion. As may be observed from table 1, the nitrogen balance was negative during the low sodium chloride period when glycosuria was most marked, but was positive during the high salt period. This protein-sparing effect has been observed repeatedly in the series of experiments thus far completed. It is an additional proof of improved carbohydrate utilization.

The systolic and diastolic blood pressure levels both rose well above the upper limits of normal after the second day of high salt ingestion and remained elevated until the second day following withdrawal of the salt. The systolic level was increased approximately 40 per cent while the diastolic rose about 30 per cent. All of the changes recorded showed a period of lag at the beginning and at the end of the experiment, accompanying respectively the initial retention and then the gradual excretion of extra salt. The patient complained of headache on several occasions when his systolic pressure rose above 165 mm. of mercury.

Certain of the data from the foregoing experiment are presented graphically in figure 1, which is largely self-explanatory. In addition, the chart shows the ineffectiveness of adrenal cortical extract (Eschatin P.D.) as a substitute for NaCl in this relationship, when given in average-sized doses. Obviously this is not a crucial or altogether satisfactory test of this point because maximum doses were not given.

Since it at first appeared possible that the response to high sodium chloride ingestion just described might be peculiar to

patients suffering from abnormal salt craving, similar studies were made on other experimental subjects both diabetic and non-diabetic, who manifested little or no such craving.

Data are graphically presented in figure 2, which demonstrate clearly that other diabetic subjects than those marked

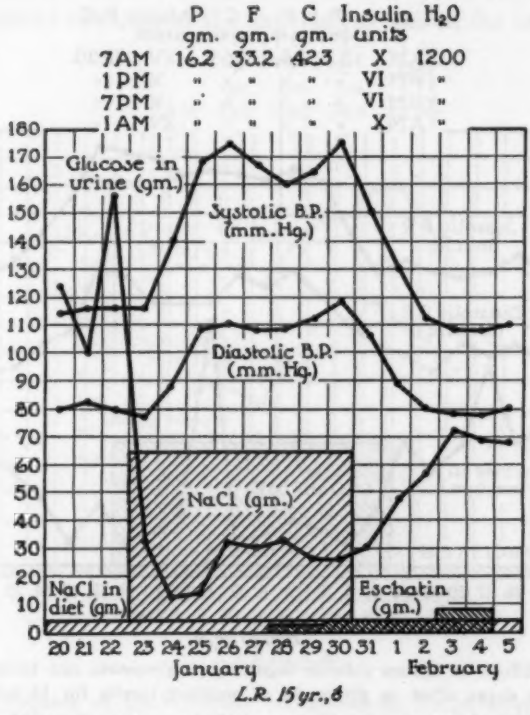

Fig. 1 Effects of sodium chloride on glycosuria and blood pressure. Non-effect of extract of adrenal cortex. L.R. severe diabetes.

by excessive salt craving respond to a sustained high intake of NaCl. It will be seen that the amount of glucose excreted per day by patient H.E. during the fore-period, namely between 40 and 70 gm. fell to between 10 and 20 gm. daily when the NaCl intake was increased from 4 to 48 gm. per day. At

the same time the systolic blood pressure rose from a basic level varying between 108 and 116 mm. of mercury to a new level ranging between 140 and 155 mm., while the diastolic level rose from between 74 and 85 to between 100 and 110 mm. of mercury. Soon after the extra sodium chloride was with-

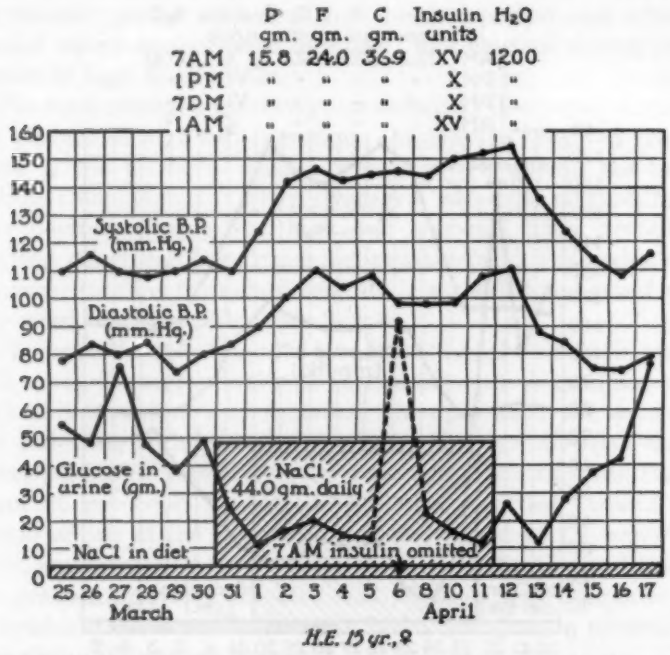
P gm.
F gm.
C gm.
Insulin units
H_2O
7AM 15.8 24.0 36.9 XV 1200
1PM " " " X "
7PM " " " X "
1AM " " " XV "
Systolic B.P. (mm.Hg.)
Diastolic B.P. (mm.Hg.)
Glucose in urine (gm.)
NaCl in diet
NaCl 44.0 gm. daily
7 A M insulin omitted
25 26 27 28 29 30 31 1 2 3 4 5 6 8 10 11 12 13 14 15 16 17
March
April
H.E. 15 yr., ♀

Fig. 2 Effects of sodium chloride ingestion on glycosuria and blood pressure. Dotted line shows effect on glycosuria of omitting insulin for 12 hours. H.E. severe diabetes.

drawn, the previous levels of glycosuria and of blood pressure were resumed.

An additional point of interest is shown in this figure, namely, that withholding of insulin for 12 hours from this moderately severe diabetic subject caused a prompt and marked increase in the degree of glycosuria without a corre-

sponding fall in the blood pressure. The effect of this withdrawal on the glycosuria curve is represented by the interrupted line.

To determine whether or not other than diabetic subjects react to overfeeding of NaCl in the foregoing manner, a single control study was made on a healthy, 14-year-old boy, L.K., who at the time of the experiment was in the late stages

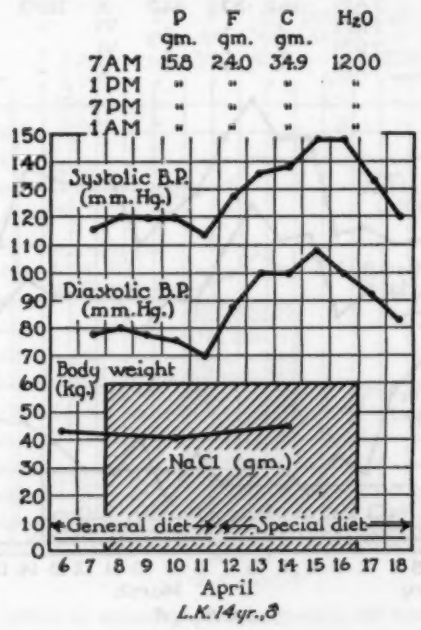

Fig. 3 Response of blood pressure to high-sodium, low-potassium intake in normal subject, L.K.

of convalescence from an operation for club foot. Since he did not have glycosuria or any disturbance of his carbohydrate metabolism, this phase of the problem could not be investigated by means of the technic employed here. It may simply be noted that he did not show any signs of hypoglycemia at the height of the salt effect. The effect on blood pressure, however, was very definitely like that of the diabetic patients.

Reference to figure 3 shows that he had no rise in either his systolic or diastolic pressure from the ingestion of 60 gm. of NaCl daily during the first 5 days while he was on the ordinary hospital diet. However, when he was given the same diet as that taken by patient H.E., namely, the simplified diet, which

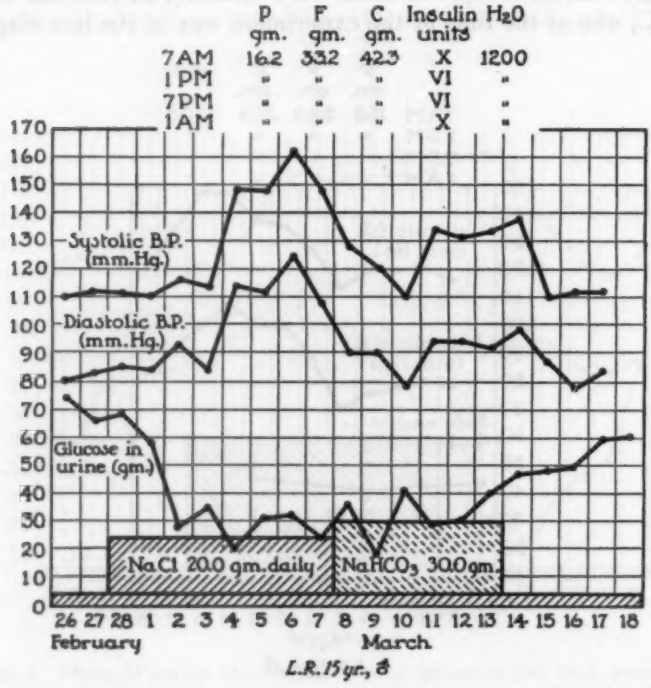
P gm.
F gm.
C gm.
Insulin units
H2O
7AM 16.2 33.2 42.3 X 1200
1 PM " " " VI "
7PM " " " VI "
1AM " " " X "
Systolic B.P. (mm.Hg.)
Diastolic B.P. (mm.Hg.)
Glucose in urine (gm.)
NaCl 20.0 gm. daily
NaHCO3 30.0 gm.
26 27 28 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
February
March
L.R. 15 yr., ♂

Fig. 4 Effects of sodium bicarbonate on glycosuria and blood pressure. L.R. severe diabetes.

was comparatively very low in K, his systolic pressure rose to between 130 and 150 mm. of mercury, while his diastolic level rose to between 90 and 108 mm. of mercury. These values gradually returned to normal following withdrawal of the extra salt.

The next objective was to determine which of the two ions, Na^+ or Cl^-, is primarily responsible for the reactions observed. The following four charts present data pertaining to this phase of the study.

An attempt to evaluate the specific effect of Na is represented by the data graphically recorded on figure 4. After eliciting the typical reaction to sodium chloride in our most

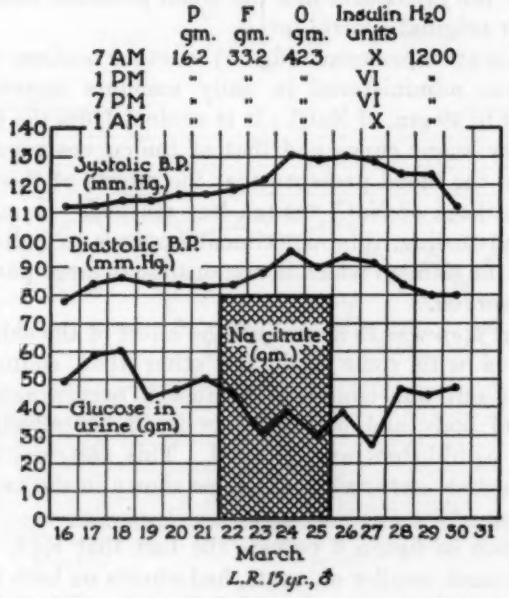

Fig. 5 Effects of sodium citrate on glycosuria and blood pressure.

responsive subject, L.R. (who manifested a craving for salt) with an extra salt intake of but 20 gm. daily, an equivalent amount of Na was substituted in the form of sodium bicarbonate. The object in giving a smaller amount of salt in this experiment than that used in the previous tests was that of avoiding, if possible, a drastic displacement of the acid-base equilibrium of the body fluids toward the alkaline side, which in itself may influence carbohydrate metabolism (Murlin and

Kramer, '16). It is seen that 20 gm. of extra NaCl daily finally resulted in a moderate decrease in the degree of glycosuria and an accompanying elevation of blood pressure after a certain amount of the salt had been retained. When the sodium bicarbonate was substituted, the effects produced by the NaCl were sustained but somewhat less efficiently. Withdrawal of the bicarbonate then resulted in a return of the values for the glycosuria and the blood pressure readings toward their original control levels.

In the next experiment (fig. 5) another sodium salt, the citrate, was administered in daily amounts approximately equivalent to 40 gm. of NaCl. It is evident from the course of the urinary sugar curve and that of the curves representing changes in the blood pressure that the effects of this salt are similar to those of NaCl though less marked. In agreement with the preceding, this experiment appears to indicate that sodium is the element which is responsible in large part for the effects observed.

The next step was to determine the effect of the chloride ion when given with some basic ion other than sodium. The potassium salt was employed because it occurs naturally in the animal body and because, like NaCl, its effects on the acid-base equilibrium are minimal. This salt was found to exert somewhat surprising effects as shown in the two following figures.

Inspection of figure 6 reveals the fact that KCl, when ingested in much smaller amounts, had effects on both the blood pressure and glycosuria of a third diabetic child, J. P., which were diametrically opposite those of NaCl. During the first 4 days shown on the figure, when she was receiving 44 gm. of extra NaCl daily, the urinary glucose varied between 20 and 30 gm. per day and the blood pressure varied between 132/98 and 150/110. Shortly after the extra NaCl was omitted, the glucose excretion rose to between 60 and 70 gm. per day, while the blood pressure fell to a level averaging around 110/78, where it remained throughout the next week. Potassium chloride was then given in gradually increasing doses over the

next 6 days, reaching a maximum of 20 gm. per day for the last 2 days. Apparently as a direct result of this, the glucose excretion increased to new levels varying from 70 to 100 gm. daily, whereas the blood pressure fell slightly below the basic level to between 86/60 and 95/62. Discontinuance of the KCl was followed within 36 hours by a return of all values to the control levels.

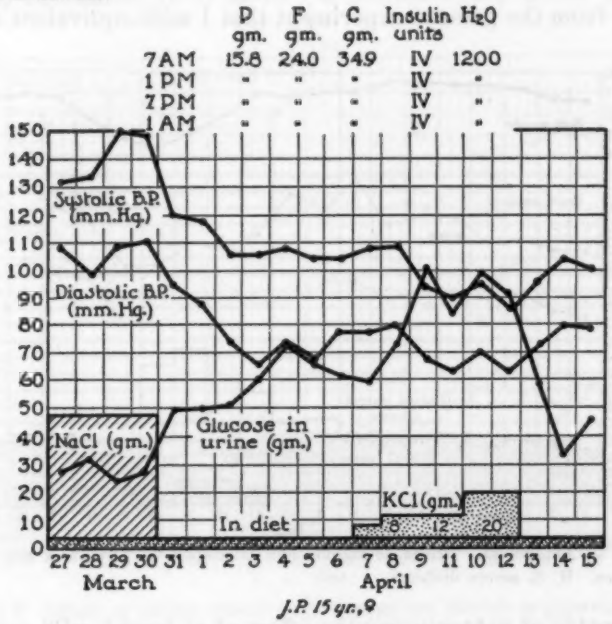
P gm.
F gm.
C gm.
Insulin units
H2O
7 AM 15.8 24.0 34.9 IV 1200
1 PM " " " IV "
7 PM " " " IV "
1 AM " " " IV "
Systolic B.P. (mm.Hg.)
Diastolic B.P. (mm.Hg.)
NaCl (gm.)
Glucose in urine (gm.)
In diet
KCl (gm.)
8 12 20
27 28 29 30 31 1 2 3 4 5 6 7 8 9 11 10 12 13 14 15
March
April
J.P. 15 yr.,♀

Fig. 6 Comparison of effects of potassium chloride with those of sodium chloride on blood pressure and glycosuria. J. P. moderately severe diabetes.

It was next attempted to ascertain whether or not Na and K exert antagonistic effects when given at the same time and, if so, in what equivalent proportions the one is capable of neutralizing the influence of the other.

Under basic conditions similar to those already described, the patient H.E. was given 40 gm. of NaCl daily over a period of 19 days. Without any other modification of the regimen,

KCl was given as follows: 12 gm. on the eleventh day, 16 gm. on the twelfth and thirteenth days and 4 gm. on the fourteenth day, after which it was withdrawn. The effects of the latter procedure are shown graphically in figure 7. It is seen that 16 gm. of KCl more than neutralized the effects of 40 gm. of NaCl. Since the potassium in 16 gm. of KCl is chemically equivalent to the sodium in but 12.6 gm. of NaCl, it would appear from the present experiment that 1 milli-equivalent of K

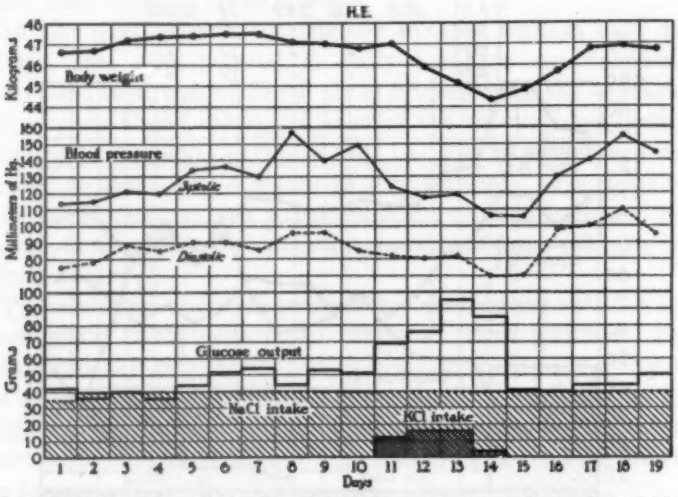

Fig. 7 Antagonistic effects of sodium and potassium on glycosuria and blood pressure. H. E. severe diabetes.

is capable of antagonizing the effect of at least 3 milli-equivalents of Na, as far as the carbohydrate metabolism and blood pressure are concerned. The difference in the effects of the two salts on the water balance of the body should be noted. It is obvious that the sodium ion is chiefly responsible for the retention of water as it is for the decrease in glycosuria and the increase in blood pressure. The decrease in glucose output as a result of NaCl ingestion was far less striking in this than in the previous experiment on H.E. (fig. 2), apparently because she was suffering from a mild upper respiratory infection during the first week of the present experiment.

The studies presented thus far, with the exception of that on one normal subject, were all carried out on severely diabetic patients who required insulin therapy. In order to determine the specific effects of the various salts without this complicating factor, the next experiment was carried out on D.R., a 14-year-old girl, whose diabetic state could be controlled fairly satisfactorily by dietary measures alone. After a pre-

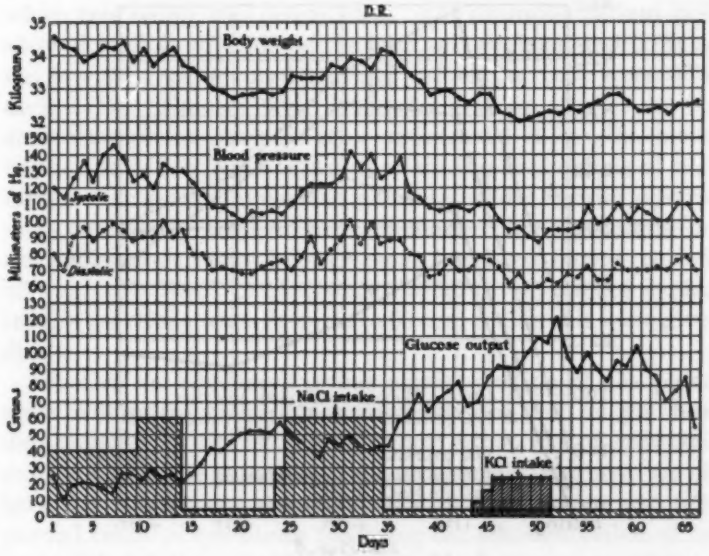

Fig. 8 Effects of sodium chloride and of potassium chloride on glycosuria and blood pressure in diabetic patient not on insulin therapy.

liminary test period, her diet was adjusted at such a level that between 50 and 75 gm. of glucose appeared in the urine daily during control periods. She was then given the salts as indicated in figure 8.

The data recorded on this figure demonstrate that the antagonistic effects of large amounts of NaCl and of KCl were qualitatively the same in this diabetic patient, who was not under the insulin therapy, as they were in the preceding cases.

The fasting serum potassium in the case of this patient was significantly lower during the period of high NaCl intake than during the control periods, the values being 27.2 mg. per 100 cc. before, 12.5 mg. during and 19.6 mg. 1 week after the high-salt period.

The next project undertaken was that of determining the effect of NaCl ingestion on the level of the blood sugar. A

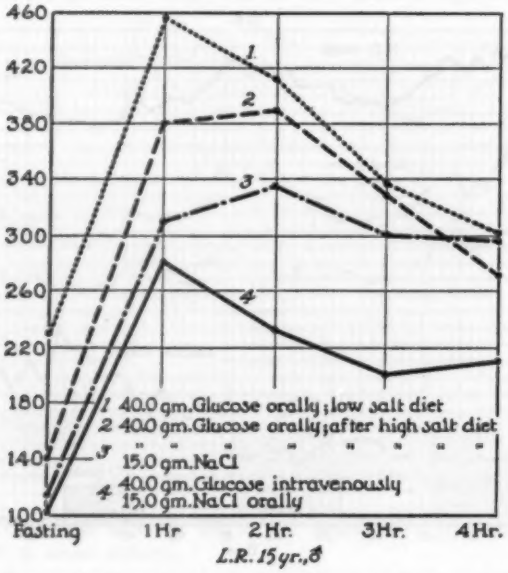

Fig. 9 Effects of sodium chloride on fasting blood sugar and on sugar tolerance curves.

series of sugar tolerance tests were made on patient L.R. under a variety of conditions as indicated on figure 9. It will be observed that all curves obtained were of the diabetic type, the ingestion of salt having no demonstrable beneficial effect under the emergency conditions of such a test. The only difference in the blood sugar findings during the control period and during the periods of high salt feeding was a tendency for the fasting values to be distinctly lower

under the latter circumstances. Nevertheless, this latter finding indicates a definite increase in the ability to utilize carbohydrates.

Additional evidence that the metabolism of carbohydrate is significantly improved by the excessive ingestion of NaCl is found in the fact that the fasting respiratory quotient was found to be slightly higher during the period of high intake than during the period of low intake in the few instances in which this factor was determined. For example, patient H.E. showed an R.Q. of 0.763 on the high salt regimen and 0.714 on the low salt, while corresponding values for L.R. were 0.747 and 0.712. Ketosis was found to develop earlier in the severely diabetic subjects after withdrawal of insulin during the period of low NaCl intake than during the periods of high intake. On several occasions patients, whose insulin dosages were sufficiently large to prevent marked hyperglycemia but not sufficient to cause hypoglycemic reactions during periods of very low NaCl intake, were found to experience typical insulin reactions when from 40 to 60 gm. of this salt were given daily.

Whether or not simultaneous administration of the potassium will prevent the latter reactions has not as yet been determined. In one extremely severe adult case of spontaneous hypoglycemia associated with acromegaly, however, we observed a steady but temporary rise in the blood sugar with partial relief of symptoms following the administration of between 24 and 48 gm. of KCl daily. Much to our surprise, no toxic symptoms were observed in the case of this man, although he finally refused to take the larger doses because of gastric discomfort. In less severe cases of spontaneous hypoglycemia, a low-sodium, high-potassium regimen might prove to be beneficial.

DISCUSSION

The foregoing data afford ample proof that the daily ingestion of sodium chloride in large amounts causes an elevation in the blood pressure and at the same time increases the ability of the diabetic subject to utilize carbohydrate. These effects are not observed if the diet is high in potassium. The effect of potassium chloride was to lower blood pressure and increase glycosuria.

A satisfactory explanation for these effects is not apparent at the present time. The effects referred to are not obtained with the small amounts of salt and water required to prevent depletion of the body's reserves. The reports of Atchley and others ('33) and Peters, Kydd, Eisenman and Hald ('33) and their co-workers have adequately described the disturbances in water and electrolyte balances which develop in uncontrolled diabetes mellitus. Kydd ('33) has emphasized the depletion of chloride in excess of other electrolytes, as if the base lost with the ketone acids might be derived from NaCl rather than from bicarbonate. He stresses the need for replacement of chloride during recovery from diabetic coma. However, in the present studies the dietary and other control factors were purposely adjusted at levels such that ketosis was extremely slight if present at all.

Since the fluid balance of the body is modified to a considerable degree and in opposite direction by the ingestion of large amounts of sodium and potassium salts, this factor must be taken into account. The decrease in glycosuria due to NaCl ingestion followed the retention of water, whereas the increase in glycosuria from potassium administration was accompanied by a mild diuresis. This observation is in keeping with the finding of Schiff and Choremis ('26) that a normal or increased hydration in the tissues favors carbohydrate utilization. It is contrary to the view of Andrews ('26) that dehydration enhances insulin action. That the glucose retained is not merely held in solution in the retained water is easily shown by comparing the small quantity so held with the total amount seen to be retained over the period of a week

(table 1). Assuming the concentration of glucose in the retained water to equal that of the blood plasma (e.g., 150 mg. per 100 cc.), the 2½ kg. of retained water would contain less than 4 gm., whereas in the course of 1 week over 300 gm. were actually retained. In those cases in which insulin therapy was employed, it might be assumed that the beneficial effect of water and salt retention in the extracellular spaces is due to the slower release of subcuaneously injected insulin. This explanation could not hold, however, in diabetics not on insulin therapy. Various data are presented which indicate that glucose is more efficiently oxidized and probably more readily stored as glycogen when large amounts of NaCl are ingested. The protein-sparing effect, fasting respiratory quotient measurements and lessened tendency to development of ketosis, as well as the decrease in glycosuria and hyperglycemia, all point in this direction.

Reference to the literature of the pre-insulin era reveals the fact that other observers obtained similar results from feeding large amounts of sodium salts. For example, Murlin and Kramer ('16) found that sodium hydroxide and carbonate increased in utilization of glucose in diabetic dogs while Murlin and Craver ('16) and Underhill ('17) observed marked decreases in glycosuria in diabetic patients following the administration of alkali. These workers attributed the effects observed to changes in the acid-base equilibrium. Beard ('18) found little or no effect from the giving of sodium bicarbonate, but observed that two of his diabetic patients, who voluntarily took increased amounts of sodium chloride and water, experienced increases in their carbohydrate tolerance. No reference is made in these papers to potassium or to the effect on blood pressure.

More recent studies on the effect of acid-base balance on carbohydrate metabolism are somewhat contradictory. Thompson, Mitchell and Kolb ('33) found in normal subjects that acid administration was followed by diminution of carbohydrate tolerance as indicated by blood sugar curves, while alkalosis had little effect. In a series of short-time experiments on normal children, Johnston and Maroney ('35) more

recently found the oxidation of dextrose to be accelerated on the acid side of neutrality and inhibited on the alkaline side. They also found the oxidation of dextrose to be depressed by the ingestion of sodium chloride and water during the short time of their retention in the body, but found it to be accelerated when the salt and water were released. Of course, these acute experiments on normal subjects cannot be compared directly with our prolonged experiments on diabetic patients. It is unlikely that the neutral NaCl or KCl, as given in our longer experiments, caused any important shift in the acid-base equilibrium.

The somewhat surprising discovery, that potassium exerts effects directly antagonistic to those of sodium, suggests the possibility that the beneficial influence of high sodium intake on carbohydrate metabolism in some way is dependent upon this interrelationship. It has been reported that serum potassium is characteristically elevated in diabetes mellitus. Rathery and Bertoliatti ('34) for instance, found values averaging 35 per cent above normal in seven cases of diabetes not on insulin therapy and 22 per cent above normal in seven insulin-treated cases. Harrop and Benedict ('24) found potassium as well as inorganic phophorus and glucose of blood to be reduced by insulin in normal and diabetic subjects. Briggs and co-workers ('23) found a decrease of 24 per cent in the serum potassium in fasting dogs following the administration of insulin in large doses. Harrop and Benedict suggest that the potassium may enter into the intermediary metabolism of carbohydrate in association with the phosphorus-carbohydrate combination. The few potassium determinations made thus far in our studies indicate that high NaCl ingestion results in a significant decrease in serum K, similar to that occurring after institution of insulin therapy. This phase of the problem is still under investigation. One case is cited above in which severe spontaneous hypoglycemia appears to have been temporarily controlled by administration of KCl in large doses.

The mechanism by which sodium salts result in an increase and potassium salts in a decrease in arterial pressure is unknown. Purely mechanical factors, such as a possible change in plasma volume and change in resistance to blood flow due to accumulation or depletion of fluid in the interstitial fluid reservoir of the body after excessive ingestion of the various salts, may play some role. Measurements of blood volume were attempted in a few instances but the results were inconclusive. Some specific effects either direct or indirect on the arterioles or the kidneys are more likely the important factors. While the observations of McLester ('22), Mosenthal and Short ('23) and others failed to confirm the claims of Allen ('22) that a 'salt free' diet results in a lowering of blood pressure in arterial hypertension, the potassium content of the diets used by the different workers may have differed sufficiently to account for their obtaining conflicting results. We are attempting at the present time to determine the effect of low-sodium, high-potassium diets on young subjects with hypertension.[2]

The possible involvement of certain nervous and endocrine factors are also being investigated by means of animal experiments. Because of the relationships of pituitary and adrenal functions to blood pressure and to the water, mineral and carbohydrate metabolism these glands are receiving first attention.

The cause of marked salt craving in two of our diabetic patients remains obscure. An underlying disturbance in the potassium metabolism was suggested by a violent, anaphylactic-like reaction experienced by L.R. when given potassium chloride in much smaller dosage than that taken without any ill effect whatsoever by other subjects studied.

Studies on the blood changes and on the mineral balances will be presented in subsequent reports.

[2] After the present paper was set up for printing, the authors discovered a paper by W. L. T. Addison (Can. Med. Assoc. J., 1928, vol. 18, p. 281) describing effects of Na and K salts on the blood pressure of adult patients with arterial hypertension, which were similar to those recorded here for non-hypertensive children.

SUMMARY

1. The effects of ingesting excessive quantities of sodium and potassium salts on the blood pressure and carbohydrate metabolism have been studied in one non-diabetic and four diabetic children. Repeated tests were made in the case of each diabetic subject. While the most striking effects were observed in the case of one particular diabetic, who consistently required between 60 and 90 gm. of NaCl daily to satisfy his craving for salt, all patients submitted to the tests responded in like manner.

2. The daily ingestion of between 1 and 2 gm. of NaCl per kilogram of body weight resulted within a period of from 2 to 4 days in a gain of from 4 to 5 per cent in body weight and an increase in both systolic and diastolic blood pressure to new plateaus between 30 and 50 per cent above the control levels. These higher pressures were maintained so long as the salt was taken.

3. In addition to this effect on blood pressure, the excessive ingestion of NaCl was found to cause a marked reduction in the degree of glycosuria in the diabetic subjects studied. The effects were qualitatively the same in one mildly diabetic patient not given insulin as in those patients who received insulin at regular 6-hour intervals.

4. The fasting blood sugar was found to range at lower levels after a few days of the high NaCl ingestion than during the foreperiod, the patient's food intake, insulin dosage and activity remaining unchanged. The fasting R.Q. was slightly higher during the period of high NaCl intake than previously. The shape of the sugar tolerance curve, however, was not measurably influenced by salt ingestion.

5. In severe diabetes, ketonuria appeared earlier after withdrawal of insulin when the NaCl intake was low than when it was very high.

6. With a constant protein intake estimated to maintain nitrogen balance under ordinary conditions the nitrogen output exceeded the intake during periods of low NaCl intake. During periods of high NaCl intake, however, the nitrogen balance became positive.

7. Typical insulin reactions were found to occur at times during the period of high NaCl intake in patients receiving insulin in dosages found during the low-salt periods to be sufficient to completely prevent glycosuria.

8. Sodium bicarbonate and sodium citrate when given in amounts with equivalent sodium values had similar though less marked effects than those of NaCl.

9. The maximum effects of NaCl on both the B.P. and the carbohydrate metabolism were obtained only when the patients were given simplified diets which were low in potassium. Ordinary diets, high in potassium, either prevented or greatly lessened the effects of the NaCl.

10. Potassium chloride, when given in doses of 10 to 20 gm. daily along with a simplified diet low in sodium, resulted in a slight fall in both systolic and diastolic blood pressure and at the same time a significant increase in the degree of glycosuria, thus exerting effects diametrically opposite those of the sodium salt.

11. When the two salts were given simultaneously one part of potassium was found to completely abolish the effects of at least three chemically equivalent parts of sodium.

12. A small number of serum potassium determinations have been made. The high NaCl intake depresses the serum potassium.

CONCLUSIONS

1. When ingested in amounts varying between 1 and 2 gm. per kilogram of body weight daily, sodium chloride exerts a favorable influence on the carbohydrate metabolism of diabetic children taking simplified diets low in potassium. This effect is observable usually on the second or third day.

2. At the same time both the systolic and diastolic blood pressure levels are elevated significantly.

3. Sodium appears to be chiefly responsible for these effects since other salts of this element as well as the chloride exert similar, though less marked, effects.

4. Potassium chloride has diametrically opposite effects on both glycosuria and blood pressure.

5. In terms of chemical equivalents, potassium completely antagonizes the effects of sodium when given simultaneously in amounts as little as one-third that of sodium.

6. The physiological mechanisms involved in these reactions are at present obscure but are being further investigated.

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